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Translational Pancreatic Cancer Research

From Understanding of Mechanisms to Novel Clinical Trials

Humana Press

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Editors

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Preface

Recent years have yielded significant progress in better understanding the pathobiology of pancreatic cancer. As a result, novel biomarkers have emerged, as have potentially effective new therapies. Translation of these results into daily clinical practice has been particularly challenging in pancreatic cancer, and large-scale, multinational efforts are only emerging.

This text has been designed in a multi-disciplinary approach to present how research results can be translated into clinical trials. It starts out with parts on variants of pancreatic cancer, precursor lesions and groups of people at risk to developing the disease. There is a particular focus on intraductal papillary mucinous neoplasia as a large-scale clinical challenge in pancreatology. This is followed by a part on (early) diagnosis, biomarkers and stratification. Here, there is a focus on various approaches to biomarker development which will be important both as prognostic and predictive tools. There is hope that the results of such research may in the near future translate into meaningful tools to aid clinical decision-making. This holds particularly true for the rapidly emerging field of multimodality and perioperative treatment of resectable, borderline-resectable and locally advanced pancreatic cancers.

Finally, there is a large section on personalized treatment approaches. As a starting chapter, preclinical models of pancreatic cancer are described, followed by chapters on stromal, epigenetic and metabolism targeting as promising approaches to be translated into early phase clinical trials. Finally, there are three chapters dealing with approaches that are close to be implemented in clinical practice or are already being tested in (early) clinical trials. These include approaches targeting the immune systems and strategies to overcome immunotherapy resistance, phase 1 clinical trials and translational approaches in surgical treatment.

Written by experts in each of the fields, these texts will not only give an overview of ongoing research efforts but will also provide an outlook towards future directions. Integrating information both from basic and clinical research, we hope that

this book – through demonstrating pathways to better understanding pancreatic cancer and current approaches to translating these into clinical practice – will be used to conceive smart, more personalized treatment schemes.

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Part I
PDAC Variants and Risk of Disease

Chapter 1
Subtypes of Pancreatic Adenocarcinoma

Luisa Ingenhoff, Lena Häberle, and Irene Esposito

Pancreatic cancers of exocrine origin are mostly represented by pancreatic ductal adenocarcinoma (PDAC) [1]. PDAC is an epithelial neoplasm with a ductal phenotype, which is reflected by strong and diffuse expression of ductal cytokeratins (CKs), such as CK7 and CK19. A few histopathological variants of PDAC are recognized and distinguished on the basis of morphology and marker profiles according to the WHO criteria [2]. PDAC subtypes partially reflect different carcinogenesis pathways, i.e., the development from different precursor lesions following different molecular pathways. Although some of these subtypes display a different biological behavior and harbor a different prognosis, the clinical relevance of such subclassifications remains limited. In particular, a correlation between morphologic and recently identified molecular subtypes is still lacking.

Tumor heterogeneity was first described in association with macroscopic and microscopic observation. Intertumor heterogeneity refers to the histological appearance of different tumors (i.e., of different patients). Intratumor heterogeneity focuses on different growth patterns, cytological characteristics, grade of differentiation, and stromal characteristics in different areas of the same tumor [3]. There are several factors determining phenotypical intratumor heterogeneity: epigenetics, hierarchical organization of cancer cell population, and heterogeneity in the microenvironment (pH, hypoxia, modulation of cell signalling, interaction between stromal and tumor cells) [4, 5]. Tumor heterogeneity is not limited to morphological features of the tumor, and genomic tumor heterogeneity exists. In PDAC, tumor heterogeneity is particularly distinct compared to other human cancers and possibly represents a prominent contributor to drug resistance and therapy failure [4, 5].

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PDAC and Morphological Subtypes

Classical PDAC (Pancreatobiliary Type)

PDAC usually presents as a white-yellow firm mass infiltrating the normal, soft, lobular structure of the pancreas (Fig. 1.1). Cystic areas may occur, usually in the form of retention cysts, sometimes being part of the tumor or displaying precursor lesions, rarely because of necrosis and/or hemorrhage. Most PDACs (70%) are located in the head of the pancreas as solitary lesions with a mean size of about 3 cm [6]. This gross aspect is usually common to most subtypes of PDAC; large areas of necrosis and hemorrhage are more common in poorly differentiated tumors. Conventional PDAC forms glandular, duct-like structures infiltrating the pancreatic parenchyma. Tumor cells are cuboidal to tall columnar and usually produce mucins of sialo-type and sulfated acid-type that accumulate in the cytoplasm or in the lumina and can be highlighted by the Alcian-blue periodic-acid-Schiff (AB-PAS) stain. A prominent clear cell differentiation is often seen. Ductal cytokeratins (CK7,

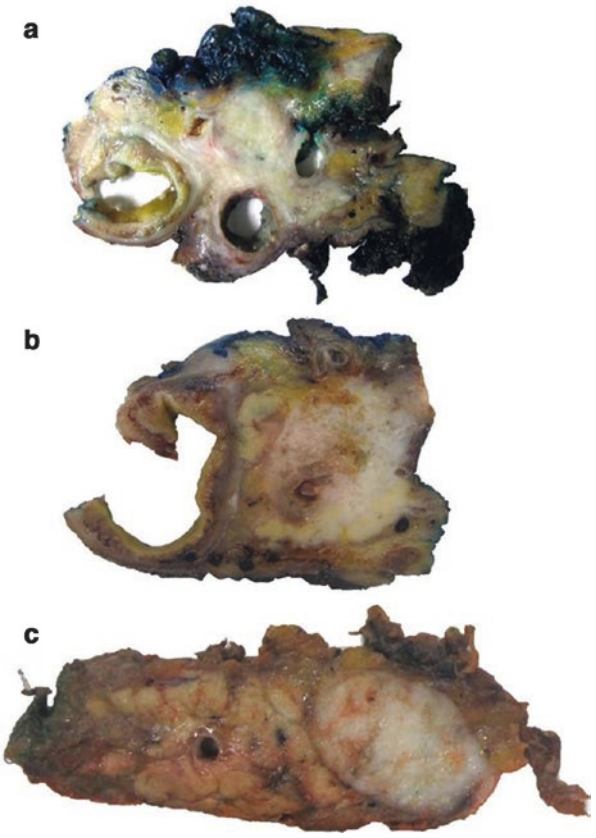


Fig. 1.1 Gross morphology. (**a**) Classical ductal adenocarcinoma of the head of the pancreas presenting as a solid, white-yellowish mass. (**b**) Colloid carcinoma of the head of the pancreas with small, cystic, mucinous areas. (**c**) Adenosquamous carcinoma of the tail of the pancreas, macroscopically not distinguishable from classical PDAC

CK8, CK18, and CK19) and the mucin proteins MUC 1, MUC 4, and MUC5AC are positive in most cases. CK20 expression is observed in about 30–75% and does not necessarily reflect an intestinal differentiation [7]. Moreover, CEA, CA19–9, and CA12.5 (MUC 16) are expressed in about 92%, 94%, and 48%, respectively [8–10]. Furthermore, about 75% of PDAC show strong expression of p53 [11, 12], which correlates with mutation of the *TP53* gene, and 55% display loss of SMAD4/DPC4 protein, also correlating with alteration of the corresponding gene [13].

Classical PDAC usually shows a quite high level of intratumoral heterogeneity concerning histological grading and pattern of growth (Fig. 1.2). The grading is assessed according to the criteria of the WHO. Briefly, *well-differentiated* PDACs display a tubular architecture with minimal nuclear enlargement, intact or slight reduced mucin production, and rare mitoses (up to 5/high-power field, HPF) [2] (Fig. 1.2a). *Moderately differentiated* PDAC shows more medium-sized duct-like structures as well as polymorph small tubular glands (Fig. 1.2b). Nuclear size, structure, and shape are more variable. Mitoses are observed more frequently

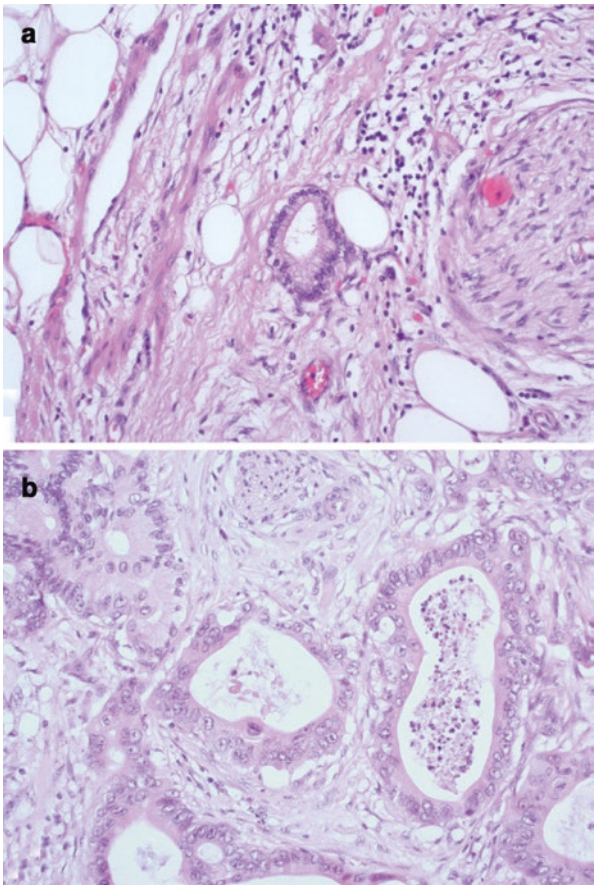
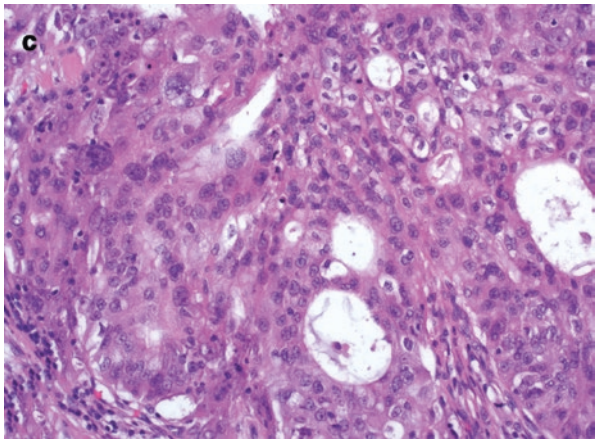


Fig. 1.2 Histology and grading. (**a**) Well-differentiated PDAC with a tubular architecture and minimal nuclear enlargement, HE 20×. (**b**) Moderately differentiated PDAC with medium-sized tubular structures and polymorph small tubular glands, as well as an abundant desmoplastic stromal response, HE 20×. (**c**) Poorly differentiated PDAC with a solid sheet structure, individual cell budding, and almost no desmoplastic stromal response, HE 20×

Fig. 1.2 (continued)



(5–10/HPF). Well- and moderately differentiated PDACs are typically accompanied by an abundant desmoplastic stromal response, which consists of dense fibrosis with activated fibroblasts and myofibroblasts, as well as leucocytes. *Poorly differentiated* PDAC is characterized by a solid sheet structure, sometimes with dense small polymorph glands with higher mitotic activity (>10/HPF) and individual cell budding (Fig 1.2c). Necrosis and hemorrhage are more common, whereas the desmoplastic stromal reaction is usually less developed to absent [2]. Tumor grading represents one of the most important prognostic indicators in PDAC [14], underlying the importance of an accurate evaluation of this parameter. This task can be particularly difficult to accomplish due to the high degree of intratumoral heterogeneity. For instance, in the periphery of the tumor, often in areas of infiltration of surrounding tissues, less differentiated areas may be present. Conventionally, the highest (=poorest) grading is assigned in the tumor classification; however, it may be useful to describe and semi-quantify any relevant component for better clinical correlation, especially concerning therapy response. Among the growth patterns, in addition to the classical tubular form, cribriform, gyriform, complex, micropapillary, large duct and papillary patterns have been described, which share the same genetic profile of the classical PDAC and appear to have no prognostic significance [15].

In addition to the above described growth pattern, homogenous variants of PDAC, defined as those containing at least 30% of a distinct histologic pattern, also exist. They include adenosquamous, colloid, undifferentiated (with or without osteoclastic giant cells), medullary, hepatoid, and signet ring cell carcinomas [2]. Many of these variants display the same genetic profile as the classical PDAC; however, some peculiarities concerning genetics and development from specific subgroups of precursor lesions, as well as regarding prognosis, exist and are briefly outlined in the following.

Adenosquamous carcinomas represent up to 10% of PDAC and have a worse prognosis compared to classical PDAC with a median survival of 7–11 months and a 3-year survival rate of 14% after surgery [2, 16–19] (Table 1.1). This variant

Table 1.1 Variants of pancreatic ductal adenocarcinoma

PDAC variant (frequency)	Histomorphology	Immunohistochemical/ molecular characteristics	Prognosis
Conventional PDAC[a] (85%)	Glandular, duct-like patterns Mucin production intracellularly and/or luminally (AB-PAS) Desmoplastic stroma	CEA+, CA19-9+, CA125+, p53+, SMAD4-	Poor (overall survival rate 6%) [40]
Adenosquamous carcinoma[a] (<10%)	Ductal as well as squamous (at least 30%) differentiation Ductal component: Similar to conventional PDAC Squamous component: Sheet-like tissue with polygonal cells, keratinization	Squamous cells: p53+, p63+, p40+, CK5/6+, p16-, SMAD4-	Poor (median survival time 7–11 months)
Colloid carcinoma[a] (2%)	Large, well-demarcated tumor masses with large extracellular mucin pools partially lined by atypical epithelial cells Associated with an IPMN of intestinal-type differentiation	CDX2+, MUC2+ High frequency of GNAS1 mutation	Good (5-year survival rate up to 85%)
Undifferentiated carcinoma[a] (<1%)	Extensive loss of differentiation Minimally cohesive, scant stroma Nuclear pleomorphisms High mitotic rate Variants: Sarcomatoid, pleomorphic, rhabdoid	High level of mutant KRAS allele-specific imbalance Rhabdoid variant: Often KRAS wild type	Poor (5-year survival rate 15%) [41]
Undifferentiated carcinomas with osteoclast-like giant cells[a] (<1%)	Highly pleomorphic, round to spindle-shaped mononuclear neoplastic Non-neoplastic reactive, multinucleated, large histiocytic giant cells often in areas of hemorrhage/necrosis	Often accompanied by MCN or in situ PDAC	Good (5-year survival rate 60%)

(continued)

Table 1.1 (continued)

PDAC variant (frequency)	Histomorphology	Immunohistochemical/ molecular characteristics	Prognosis
Hepatoid carcinoma[a] (<1%)	Hepatocellular differentiation Large polygonal cells with abundant eosinophilic cytoplasm May be accompanied by conventional PDAC, acinar carcinoma, or neuroendocrine neoplasm	AFP+, HepPar1+, CEA+, CD10+ Transposon-induced Fign mutation found recently	Unknown
Medullary carcinoma[a] (<1%)	Poorly differentiated, scarce gland formation Pushing borders Syncytial growth pattern Tumor tissue infiltrated by CD3+ lymphocytes	Loss of expression of DNA mismatch repair genes and microsatellite instability Sporadically or in lynch syndrome	Unknown
Signet ring cell carcinoma[a] (<1%)	Mucinous differentiation Poorly cohesive, individual neoplastic cells with intracytoplasmic mucin accumulation		Poor
Tubular carcinoma (unknown)	Well-differentiated open tubules	Scarce mutational events	Very good

[a]Listed in the WHO classification

displays a ductal as well as a squamous differentiation (Fig. 1.3a, b). The WHO definition of adenosquamous carcinoma requires at least 30% of the tumor mass to be squamous, whereas even a minimal ductal component warrants the classification of a given PDAC as adenosquamous variant [2]. Squamous cells are usually easily recognized by their eosinophilic cytoplasm with prominent intercellular junctions and, in some cases, by keratinization. In doubtful cases, p63 and/or p40 immunostaining can be applied to highlight a squamous component [20, 21]. Molecular studies, including a recent whole-genome and whole-exome sequencing study of a series of 17 adenosquamous carcinomas, have revealed numerous similarities to classical PDAC, the only exception being the higher frequency of *TP53* mutations [22].

Undifferentiated carcinomas represent less than 1% of PDAC and are characterized by an extensive loss of differentiation accompanied by severe cellular and nuclear pleomorphism [16]. Several subtypes of undifferentiated carcinomas (e.g., sarcomatoid, pleomorphic, rhabdoid) are recognized with distinct morphologic features but have common clinical characteristics (Fig. 1.3c, d). Undifferentiated carcinomas have been shown to bear a high level of mutant *KRAS* allele-specific imbalance compared to classical PDAC, which correlate with aggressive clinical behavior [23, 24]. The rhabdoid variant often has a *KRAS* wild-type status and bears on the other hand alterations of the *SMARCB1* gene

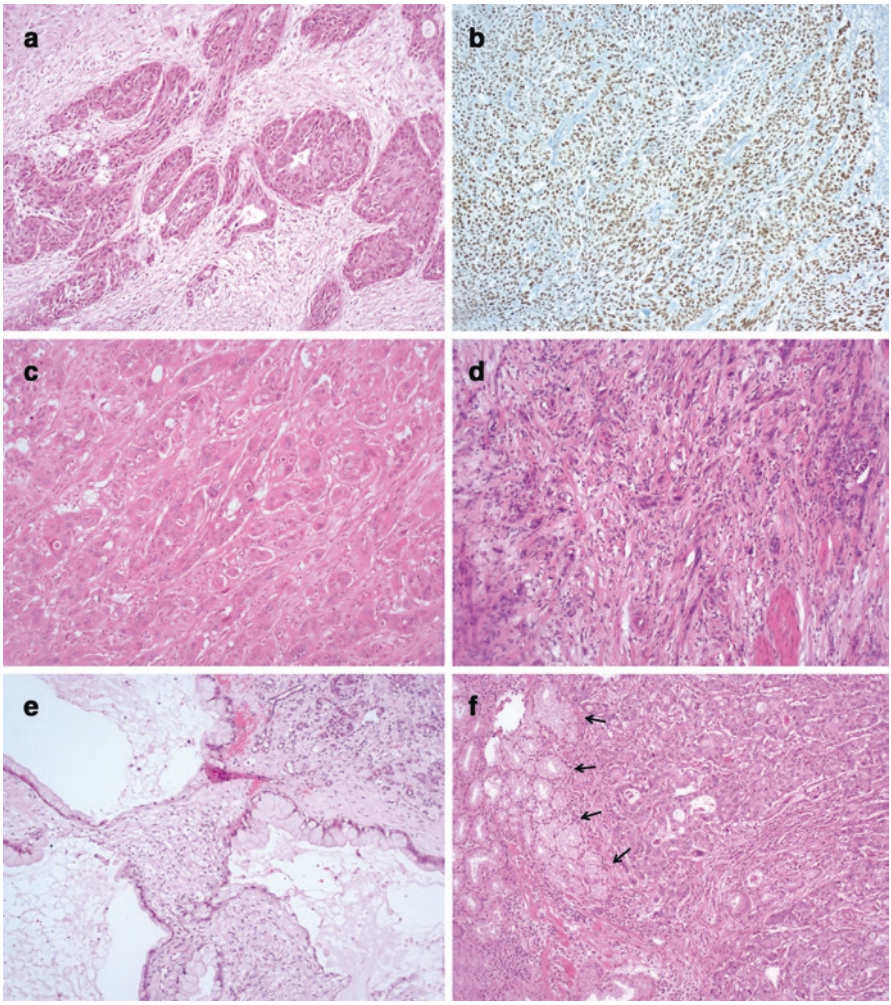


Fig. 1.3 Variants of PDAC. (**a**) Adenosquamous PDAC showing squamous as well as ductal tumor components accompanied by an abundant desmoplastic stromal response, HE, 10×. (**b**) Squamous component in adenosquamous PDAC is positive for p40, 10×. (**c**) Anaplastic pleomorphic PDAC with giant tumor cells growing in a solid sheet pattern, HE 10×. (**d**) Anaplastic PDAC, sarcomatoid variant, showing spindle-shaped sarcoma-like cells, HE 10×. (**e**) Colloid carcinoma showing mucin pools partially lined with atypical cuboidal epithelium, HE 10×. (**f**) Medullary carcinoma showing poorly differentiated tumor cells growing in a syncytial pattern and "pushing borders" phenomenon (arrows), HE, 10×

with loss of expression of the corresponding protein at the immunohistochemical level [25].

Signet ring cell carcinoma is very rare variant of cancer with mucinous differentiation and aggressive clinical behavior. It displays poorly cohesive, individual neoplastic epithelial cells with intracytoplasmic mucin accumulation [2].

A few homogeneous variants of PDAC show a better prognosis compared to the conventional pancreatobiliary subtype. However, survival data are for some entities too limited to allow confident statements.

Undifferentiated carcinoma with osteoclast-like giant cells is characterized by the presence of multinuclear histiocytic giant cells often residing in areas of hemorrhage and necrosis. Although previous data have ascribed a particularly aggressive behavior of this variant, a recent large series has identified relevant clinical peculiarities of this PDAC subtype, such as the frequent occurrence in a younger population compared to classical PDAC (mean age 57 vs. 70 yrs.) and a better prognosis with a 5-year overall survival of 60% [26]. An interesting aspect is the peculiar association with mucinous cystic neoplasms or PanIN (pancreatic intraepithelial neoplasm) but not with other PDAC precursors [27].

Colloid (mucinous non-cystic) carcinoma represents up to 2% pancreatic cancers and is usually associated with main duct intraductal papillary mucinous neoplasms of the intestinal subtype. Colloid carcinomas usually form large, well-demarcated tumor masses characterized by large extracellular mucin pools partially lined by atypical epithelial cells [16] (Fig. 1.3e). In addition, groups of tumor cells can be found floating in the mucin pools. Intestinal-type IPMNs (intraductal papillary mucinous neoplasms) are characterized by the expression of markers of intestinal differentiation, like MUC2 and CDX2, which can be also detected in the cells of colloid carcinoma but are uncommon in other PDAC variants [28]. Both intestinal IPMN and colloid carcinomas are characterized by a high frequency of *GNAS1* mutations, underscoring the existence of an intestinal-type progression model in addition to the conventional, *KRAS*-driven pancreatobiliary carcinogenesis [29]. Mucinous carcinomas have a good prognosis with a 5-year-survival rate up to 83% [30].

Medullary carcinomas are poorly differentiated epithelial neoplasms displaying scarce gland formation. Typically, the tumor mass has "pushing" anatomical borders and shows a syncytial growth pattern with numerous infiltrating T lymphocytes (Fig. 1.3f). Medullary carcinomas can occur sporadically or in the context of Lynch syndrome and often display microsatellite instability with loss of expression of mismatch repair proteins at immunohistochemistry [31]. Their prognosis appears more favorable than that of conventional PDAC [32, 33], but the mean survival time is unknown because of its rarity [34].

Recently, a rare variant of well-differentiated tubular adenocarcinoma, morphologically resembling tubular carcinoma of the breast, has been described. This variant shows paucity of mutational events and has a very good prognosis [15].

Hepatoid carcinoma is a very rare epithelial neoplasm with a component of hepatocellular differentiation with large polygonal cells with abundant eosinophilic cytoplasms and HepPar1 immunolabeling. AFP, CD10, and CEA with canalicular pattern may be expressed [35, 36]. Hepatoid PDACs develop along different molecular pathways compared to the conventional subtype [37, 38]. These pathways, which have been partially disclosed using transposon-induced mutagenesis, include alterations of *Fign* gene in the form of *Fign* insertions demonstrated in a recent mouse model study. *Fign* insertion leads to *Fign* overexpression which was found in

hepatoid pancreatic cancer [39] . Survival data of hepatoid carcinoma are lacking so far (Table 1.1) [40, 41].

Stromal Heterogeneity in PDAC

An abundant stroma, consisting of various extracellular matrix proteins and cancer-associated (myo-)fibroblasts, termed pancreatic stellate cells (PSCs), is a hallmark of PDAC. While some studies imply that the stroma can have a protective effect in PDAC [42, 43], many data suggest that the stromal reaction promotes the aggressive tumor biology of PDAC as well as its chemoresistance [44–46].

It has been shown that both the desmoplastic stroma and PSC are characterized by marked heterogeneity. The stroma itself can be characterized into histomorphological subgroups according to its composition, e.g., in dense (mature), intermediate, and loose (immature) stroma. Some studies imply that a dense collagen-rich stroma is linked to a better outcome of PDAC patients, compared to a loose mucin-rich stroma characterized by dynamic stromal remodeling, which is correlated with poorer prognosis [47–49]. In addition, the heterogeneous expression of PSC markers in PDAC tissue specimens suggests the presence of PSC at different levels of activation or differentiation or even the presence of different PSC subpopulations [50]. Here, the presence of α-SMA-positive PSC seems to be correlated with worse survival [47, 50, 51].

While these histomorphological subtypes of PDAC stroma have been recapitulated by molecular analyses in part [52], an association of these stromal subtypes to the various histomorphological epithelial subtypes has not been established yet.

PDAC and Molecular Subtypes

With high-throughput techniques becoming more and more readily available, a new concept of molecular subtyping of PDAC has emerged in recent years.

In 2011, Collisson and colleagues proposed three molecular subtypes of PDAC: the *classical*, the *quasi-mesenchymal***,** and the *exocrine-like subtype* [53].These subtypes seem to be relevant for survival, with the classical subtype displaying the best prognosis and the quasi-mesenchymal subtype the worst [53]. Moreover, Collisson's subtypes are suggested to be correlated with therapy resistance and sensitivity [53].

Five years later, Bailey et al. suggested the existence of four molecular PDAC subtypes, which overlap in part with the subtypes proposed by Collisson's group: the *squamous subtype*, corresponding to Collisson's quasi-mesenchymal subtype, the *aberrantly differentiated endocrine exocrine (ADEX) subtype*, recapitulating Collisson's exocrine-like subtype, the *pancreatic progenitor subtype*, which seems to be linked to Collisson's classical subtype, and, lastly, the *immunogenic subtype* [54].

In addition to identifying a more favorable "classical" and a prognostically adverse "basal-like" epithelial *subtype* of PDAC, Moffitt and colleagues also proposed two molecular subtypes of PDAC stroma: the "normal" and the "activated" PDAC stromal subtype, with the "activated" subtype being linked to worse prognosis [52].

Taking into consideration the mutational burden, the histomorphological stroma subtype, and the immune infiltrate, the group around Knudsen defined four new molecular PDAC subtypes. *Cluster 1* includes PDACs with low mutational burden, low stromal volume, immature stromal type, and a high number of macrophages ("mutationally cold"), while *Cluster 2* describes PDACs with high mutational activity and high levels of all immune cell types ("hot"), *Cluster 3* is defined as "mutationally active," displaying a high mutational burden, an intermediate stromal type, higher numbers of tumor-infiltrating lymphocytes (TILs), and peritumoral lymphocytes but relatively low levels of macrophages, and *Cluster 4* includes PDACs with low mutational burden, high stromal volume, mature stromal type, and low immune cell levels ("cold") [49]. In this study, Cluster 4 PDACs seem to display improved overall survival compared to all other "immunosubtypes" of PDAC [49].

Although these subtypes described by different authors seem to display some similarities between each other, there is no complete overlap. This may be partially due to methodological imperfections of the studies performed so far. PDAC characteristically consists of dispersed tumor glands embedded in a prominent desmoplastic stroma. This may have led to the contamination of tumor tissue samples with stromal cells during microdissection. Very recently, evidence has also been found that that Collisson's exocrine-like subtype (Bailey's ADEX subtype) may have been a result of contamination of tumor tissues with normal acinar cells of the pancreas [55].

Some molecular subtypes can be recapitulated by immunohistochemistry. For example, immunohistochemical positivity for CK81 identifies PDACs of Collisson's quasi-mesenchymal, Bailey's squamous, and Moffitt's basal-like subtype, while HNF1alpha positivity identifies "non-quasi-mesenchymal," "non-squamous," and "non-basal-like" PDACs [56]. The relevance of these immunohistochemical subtypes for survival has been validated in different patient cohorts, with HNF1alpha-positive PDACs showing the best survival and CK81-positive PDACs the worst [56]. This seems like a big step in integrating molecular subtyping into routine diagnostics. However, the correlation between molecular and immunophenotypical subtypes and histomorphological subtypes is still lacking in PDAC. Most surprisingly, even though the adenosquamous histomorphological variant of PDAC is also associated with especially poor prognosis, no correlation could be established between the histomorphological (adeno-) squamous phenotype and the molecular quasi-mesenchymal/squamous/basal-like subtype yet. Nevertheless, certain links between histomorphological and molecular features of PDAC have been found in the past. For example, *KRAS* mutations are significantly more common in classical PDACs than in its histomorphological variants [15].

While establishing clear associations between histomorphology and molecular profiles, as it has been done in other tumor entities such as lung cancer, proves

utterly challenging in PDAC, this still seems to be the next step to take in order to translate molecular findings into viable clinical applications.

Conclusion

Intra- and intertumoral heterogeneity is an emerging concept in PDAC. In addition to histomorphological subtypes, molecular subtypes, even of PDAC stroma, have been proposed. The prognostic and therapeutic relevance of PDAC subtyping is currently under investigation and has delivered promising results. However, the WHO classification has not yet adapted the whole morphological and molecular spectrum and is based mainly on tumor morphology and marker profiles. A correlation between histomorphologic and molecular subtypes is still lacking.

A major task in future studies is to find consensus about the newly described molecular subtypes and to integrate them with morphological features to generate a universal classification that can be easily applied in everyday practice.

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Part II
PDAC Precursors and Early Diagnosis

Chapter 2
Surveillance and Intervention in IPMN

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More frequent use of high-quality cross-sectional imaging, increased life expectancy, and the trend for healthy individuals to undergo "health checkups," including full-body magnetic resonance imaging (MRI), have increased the detection of intraductal papillary mucinous neoplasm of the pancreas (IPMN) [1]. IPMN is a heterogeneous group of pancreatic cystic neoplasm arising from the proliferation of mucin-producing cells within the pancreatic ducts [2]. IPMN can be morphologically divided into main duct IPMN (MD-IPMN), branch duct IPMN (BD-IPMN) and mixed-type IPMN (MT-IPMN) on the basis of the anatomical distribution of duct(s) dilatation in the pancreatic gland [3, 4].

IPMN represents 20–50% of all pancreas cystic neoplasms and 1–3% of the exocrine pancreatic neoplasms [5–7]. The male to female ratio reported for IPMN in the population is 3:1 (2:1 for BD-IPMN) [8]. Surprisingly, these ratios seem to vary between countries/regions. A male predominance was observed in Korea and

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Japan, while a more even distribution between male and female was observed in the United States and in Europe. The mean age of presentation is in the fifth to seventh decade [9], and the prevalence increases with increasing age of the population [10].

Due to the potential for progression to invasive cancer, patients with IPMN are routinely monitored. The primary goal is to prevent malignancy and/or alleviate symptoms while avoiding unnecessary surgery. Currently, four guidelines, the 2015 American Gastroenterological Association (AGA) [11], the 2017 International Association of Pancreatology (IAP) [10], the 2018 American College of Gastroenterology (ACG) [12], and the 2018 European Study Group on Cystic Tumours of the Pancreas (European) [13], provide recommendations on surveillance and surgical resection based on symptoms and perceived risk of malignancy (Table 2.1).

Classification of IPMN

Radiological Classification

The morphological classification of IPMN in MD-, BD-, and MT-IPMN is based on radiological characteristics. These subtypes harbor a different risk of malignancy, and therefore each requires a specific therapeutic approach (Fig. 2.1).

MD-IPMN can be recognized by the abrupt dilatation of the pancreatic main duct and the presence of mucus together with villous neoplastic component. The dilatation of the pancreatic main duct can be segmental or along the entire duct. For resected MD-IPMN, the mean frequency of advanced neoplasia (invasive cancer or HGD) is 61.6% (range 36–100%), and the mean frequency of invasive cancer is 43.1% (range 11–82%) [14–26].

BD-IPMN is characterized by a "grape-like" dilatation of pancreatic side branch ducts. For resected BD-IPMN, the mean frequency for invasive carcinoma and high-grade dysplasia (HGD) is 31.1% (range 14.4–47.9%), and the frequency of invasive cancer is 18.5% (range 6.1–37.7%) [27–33].

MT-IPMN presents radiological characteristics of both MD- and BD-IPMN. For resected MT-IPMN, the mean frequency of HGD and invasive carcinoma is the same as for MD-IPMN.

Histological Classification

Histologically, IPMN can be divided on the basis of the epithelium in different histologic phenotypes: intestinal, gastric, oncocytic, and pancreatobiliary type. Typically, these distinctions can only be made reliably based on surgical specimens, thus limiting their value in the diagnostic process [34].

Table 2.1 Absolute and relative indications for surgical resection by 2015 AGA, 2017 IAP, 2018 European, and 2018 ACG guidelines

Guidelines	Cyst type	Absolute indications for surgery	Relative indications for surgery
2015 AGA guideline	IPMN	PD ≥ 5 mm (on MRI *and* EUS) *and* solid component *or* cytology positive for malignancy	
2017 IAP guideline	IPMN	Cytology suspicious or positive for malignancy Jaundice (IPMN related) Enhancing mural nodule (≥5 mm) PD dilatation ≥ 10 mm	Grow rate ≥ 5 mm/2 years Increased levels of serum CA 19.9 PD dilatation between 5 and 9 mm Cyst diameter ≥ 30 mm Acute pancreatitis (caused by IPMN) Enhancing mural nodule (<5 mm) Abrupt change in caliber of PD with distal pancreatic atrophy Lymphadenopathy Thickened/enhancing cyst walls
2018 European guideline	IPMN	Positive cytology for malignancy/ HGD Solid mass Jaundice (IPMN related) Enhancing mural nodule (≥5 mm) PD dilatation ≥ 10 mm	Grow rate ≥ 5 mm/year Increased levels of serum CA 19.9 (>37 U/m) * PD dilatation between 5 and 9.9 mm Cyst diameter ≥ 40 mm New onset of diabetes mellitus Acute pancreatitis (caused by IPMN) Enhancing mural nodule (<5 mm)
2018 ACG guideline	IPMN	Decided by multidisciplinary team Referral in case of: Jaundice (IPMN related) Acute pancreatitis (caused by IPMN) Increased levels of serum CA 19.9 Mural nodule/solid component PD dilatation > 5 mm Cyst diameter ≥ 30 mm Positive cytology for malignancy/ HGD	

ACG American College of Gastroenterology, *AGA* American Gastroenterological Association, *CA 19.9* cancer antigen 19.9, *EUS* endoscopic ultrasound, *HGD* high-grade dysplasia, *IAP* International Association of Pancreatology, *IPMN* intraductal papillary mucinous neoplasm, *MRI* magnetic resonance imaging, *PD* pancreatic duct

*The 2015 AGA guideline suggests to discontinue the follow-up after 5 years, if there is no change in size or characteristics of the cyst

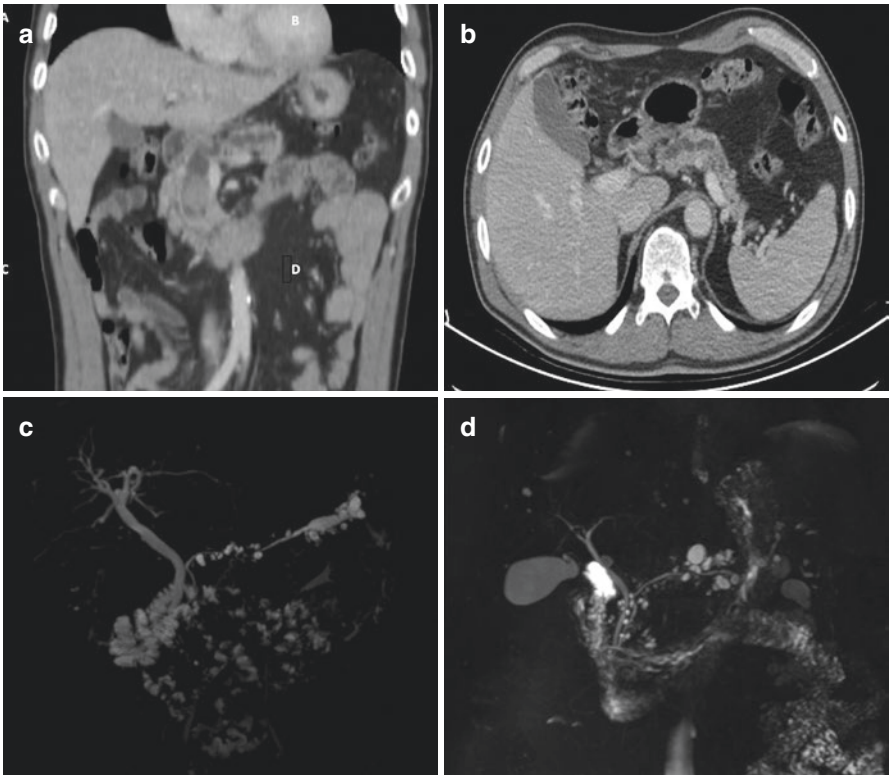

Fig. 2.1 Different types of IPMN. (**a**) BD-IPMN with slender MPD in the tail. (**b**) Both dilated MPD and BD in the pancreatic tail, image matching a MT-IPMN. (**c**) Dilated MPD in the head of the pancreas. (**d**) Image matching a MD-IPMN with solid component as a sign of a possible malignant degeneracy

Further distinction is based on cytological and architectural atypia in noninvasive IPMN. Currently, the World Health Organization (WHO) classification recommends a three-tiered system for grading of dysplasia in IPMN, from low- to high-grade dysplasia [35].

Low-grade dysplasia (LGD) is characterized by cells with oriented nuclei with small variability in nuclear size, shape, and retained polarity. *Moderate-grade* dysplasia is defined by nuclear pleomorphism, increased nucleus-to-cytoplasm ratio, and nuclear pseudostratification. *High-grade* dysplasia (HGD) features architectural complexity and marked variability in nuclear size and shape [36]. In order to improve the concordance of reporting and alignment with practical consequences, a two-tiered grading system has been proposed (low- versus high-grade dysplasia) [37].

Regarding the histological classification of IPMN, *gastric*-type IPMN is characterized by low-grade dysplasia and abundant cytoplasmic mucin that expresses *MUC-5AC*. When the gastric type has invasive characteristics and is localized in the pancreatic main duct, it is more likely a more aggressive tubular carcinoma [38].

The *intestinal epithelial* type [39] is the most common in IPMN and resembles normal intestinal epithelial cells with expression of *MUC-2* and *CDX-2*. *The pancreatobiliary*-type IPMNs express MUC-1, and in this type, cells are organized as complex papillae. This subtype is associated with invasive carcinoma in 90% of patients. The pancreatobiliary subtype is also associated with invasive tubular adenocarcinoma, and both morphology and prognosis are similar to PDAC (pancreatic ductal adenocarcinoma) [40–42]. The *oncocytic*-type IPMN is characterized by cells with abundant eosinophil cytoplasm rich in mitochondria organized in complex papillae or solid sheets and severe high-grade dysplasia [43].

IPMN can contain more than one subtype, and it is recommended to report the dominant subtype and/or the subtype exhibiting the highest degree of dysplasia. The oncocytic type occurs only in a "pure" form, without mixing with other different histological subtypes [40, 41]. A 2011 study classified 283 surgically resected IPMNs: 137 BD-IPMNs, 102 MD-IPMNs, and 44 MT-IPMNs. Among these, 139 patients had gastric type (90 patients with BD-IPMN, 34 with MD-IPMN, and 15 with MT-IPMN), 101 patients had intestinal type (28 patients with BD-IPMN, 54 with MD-IPMN, and 19 with MT-IPMN), 24 patients had oncocytic type (12 patients with BD-IPMN, 8 with MD-IPMN, and 4 with MT-IPMN), and 19 had pancreatobiliary type (7 with BD-IPMN, 6 with MD-IPMN, and 6 with MT-IPMN) [41]. These findings are supported by other studies [40, 44] and demonstrate that the gastric and intestinal subtypes are the most common and that all histopathological subtypes can be found in the three morphological imaging-based subtypes (BD-, MD-, MT-IPMN).

Intraductal tubulopapillary neoplasm (ITPN) is a rare intraductal epithelial neoplasm of the pancreas recently recognized as a distinct entity by the WHO classification in 2010. It accounts less than 1% of all pancreatic exocrine neoplasms and the 3% of intraductal pancreatic neoplasms. Compared to IPMN, they are less often cystic, typically mass forming, without overt production of mucin. ITPNs typically have uniform high-grade dysplasia, and approximately 40–50% of the cases are associated with invasive cancer [45, 46]. ITPN is often difficult to differentiate histologically from IPMN, especially the pancreatobiliary and oncocytic subtype. ITPNs showed positive for cytokeratin, CK19, MUC1, and MUC6 at the immunohistochemistry analysis [47].

Diagnosis

Symptoms

Most IPMNs do not cause symptoms. In case of symptoms, the most common are weight loss, pancreatitis, jaundice, palpable mass, and postprandial fullness according to a study from a high-volume center. Only pancreatitis and jaundice could be related to the presence of IPMN [48]. Main duct IPMN is more often symptomatic than branch duct IPMN. This can be related to the massive production of mucin in MD-IPMN;

mucin plugs may occlude the pancreatic duct and lead to acute pancreatitis with epigastric discomfort. These symptoms have been reported in approximately 25% of patients with MD-IPMN [49, 50]. The chronic obstruction of the outflow of pancreatic juice can lead to pancreatic endocrine and exocrine insufficiency and resulting diabetes, diarrhea, and steatorrhea. Jaundice can be secondary to mucin plugs in the distal bile duct or direct tumor invasion in case of malignant progression.

Symptoms, such as acute pancreatitis, jaundice, or new-onset of diabetes mellitus, are mostly associated with high-grade dysplasia or invasive carcinoma [51, 52]. These symptoms in the presence of an IPMN have been part of the IAP and European criteria in the predictive factors for malignant IPMN [9, 46, 53].

Imaging Techniques

Currently, cross-sectional imaging plays a central role in lesion detection and differentiation of IPMN. The presence and extent of IPMN can be assessed with computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS). Gadolinium-enhanced magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP) is the modality of choice, because of its superiority in identifying a connection between the MDP and the lesion and mural nodules and septations, as well as cyst differentiation [10, 54]. In addition, studies have shown that repeated exposure to ionizing radiation following CT increases the risk of malignancy. Therefore MRI/MRCP, avoiding the ionizing radiations, is the preferred method for surveillance of PCN (pancreatic cystic neoplasm) [1]. By definition, branch duct IPMNs have a communication to the main pancreatic duct that can be best assessed with either MRI (90–100%) or EUS (80–90%) [55]. For MD-IPMN and MT-IPMN, a focal or diffuse involvement of the main pancreatic duct can easily be assessed by MRI/MRCP and EUS. A systematic review reported that CT is able to correctly differentiate benign from malignant cysts with 71–80% accuracy and a presence of a communication between the cyst and the pancreatic duct with 80% accuracy; for MRI and MRCP, these were 55–76% and 96% [56]. Another systematic review including 37 studies observed a pooled 81% sensitivity and 76% specificity for risk features predictive of malignancy on CT/MRI [57]. Higher accuracy can be observed with EUS, with a 65–96% accuracy to detect benign from malignant cyst, but due to its invasive nature, it should be reserved for selected cases [58].

Cyst Fluid Analysis and Biomarkers

EUS allows fine needle aspiration (FNA) of the cyst fluid. EUS-FNA is a safe procedure. In a retrospective study in two experienced academic institutions, the complication rate of EUS in 603 patients was 2.2% with pancreatitis, abdominal pain, retroperitoneal bleeding, infection, and bradycardia as main complications [59].

A cyst fluid CEA (carcinoembryonic antigen) with a cutoff of 192–200 ng/ml [4, 60], as well as amylase, can be helpful in the differential diagnosis of pancreatic cysts and grade of dysplasia. CEA level showed to have 52–78% of sensitivity and 63–91% of specificity for identifying IPMN and MCN [13, 61, 62].

Cytology may report on low- and intermediate-grade dysplasia, high-grade dysplasia, or invasive carcinoma [63]. It is, however, common to find different grades of atypia within the same lesion; therefore the cytological examination of IPMN is not enough to assess the entire cytological pattern of the cystic lesion. Matthaei et al. [64] reported that the analysis of cells in the cystic fluid allowed to detect invasive carcinoma and HGD with 72% of sensitivity and positive predictive value (80% accuracy).

DNA-based testing of pancreatic cyst fluid seems to be a promising adjunct for the differentiation between mucinous and non-mucinous PCN, between mucinous PCNs (IPMN versus MCN), and between premalignant PCNs and those with advanced neoplasia. Many genetic mutations have been reported regarding IPMN: KRAS (~80% of IPMN), GNAS (~70% of IPMN), RNF43, PIK3CA, p16/CDKN2A, SMAD4, and Tp53 [65, 66]. The mutation of GNAS and KRAS is seen in >90% of IPMN [66, 67], and GNAS mutation is more common in intestinal-type IPMN [66, 68].

From recent genetic studies, it is clear that both invasive and noninvasive components tend to harbor identical mutations [65, 66]. In the near future, micro-RNA might be the key to distinguish IPMN from other cysts of the pancreas and even discern low-grade IPMN from high-grade dysplasia IPMN [68–70]. Moreover glycoprotein altered expression in the cystic fluid might be useful as well in differentiating IPMN with low-grade dysplasia from high-grade IPMN [71–73].

New Developments in Imaging Techniques

Recent evidence suggests that MRCP (thick and thin T2 slices, centered on the main pancreatic duct at the head and body/tail level) or CT scan with slices <2 mm width (three phases: no iodine IV contrast, arterial, and portal phases) should be used when evaluating a pancreatic cyst [1, 10]. EUS should remain a third option for those cases in whom the radiographic characterization of the pancreatic lesion is unclear [74]. Nevertheless, EUS is very useful to detect mural nodules, especially when the examination is integrated with a contrast-enhanced endoscopic ultrasound (CH-EUS) [75]. Contrast-enhanced EUS (CE-EUS) can be used to better differentiate a mucin plug and mural nodule using echo-Doppler during the examination, and even better definition can be assessed with tissue harmonic echo (THE) [76]. Nevertheless, EUS is an operator-dependent procedure that relies on the specialist's experience and ability.

More recently, a new endoscopic modality has been described, the needle-based confocal laser endomicroscopy (nCLE) that can provide a real-time in vivo optical biopsy with the use of a fluorescent dye [77]. The nCLE has been proven feasible and reliable in differentiating SCN from mucinous lesions [78–82].

The micro-forceps biopsy (MFB) is showing good results in the assessment of the nature of pancreatic cysts. The device can be inserted in a 19 gauge needle during the endoscopy procedure and allow a "micro-biopsy" from the cyst wall or septations for histological evaluation of the cyst architecture and subepithelial stroma. The MFB can be used in addition to the pancreatic cyst fluid (PCF) examination and in a recent paper by Zhang et al. [83] has proven good result in diagnosing specific type of pancreatic cyst, with consequent important implications regarding the management of the patients. The presence of epithelial stroma in the biopsy performed with the micro-forceps can help the pathologist in the differential diagnosis between MCN and IPMNs [83].

Another technique to identify and characterize pancreatic IPMNs is the peroral pancreatoscopy (POPS) [84]. The added value of this technique appears to lie in the ability to identify pancreatic duct skip lesions (reported in about 6–19% of the patients [85]) in order to reduce recurrences after pancreatic surgery [86]. In addition, POPS allows collection of pancreatic juice for cytopathological examination and for biopsy using the mini-forceps.

Clinical and Radiological Characteristics Associated with Advanced Neoplasia

Many guidelines have been published on management of pancreatic cystic neoplasms (PCNs): the IAP (2017) guideline for the management of IPMN of the pancreas [10], the European evidence-based guideline (2018) on pancreatic cystic neoplasms [54], the AGA guideline (2015) [87], and the ACG clinical guideline (2018) [88].

According to both the IAP and European guidelines, jaundice, the presence of an enhancing mural nodule ≥5 mm, the presence of a solid component, positive cytology, and a dilated PD ≥ 10 mm are highly predictive of advanced neoplasia and therefore an absolute indication for resection in surgically fit patients. According to both the 2017 IAP and the 2018 European guidelines, acute pancreatitis caused by IPMN, an enhancing mural nodule <5 mm, a dilated PD between 5 and 9.9 mm, and an increased level of serum CA19.9 without jaundice are associated with advanced neoplasia in IPMN and therefore a relative indication for surgery in patients fit for surgery.

According to the 2017 IAP guideline, a thickened or enhancing cyst wall, lymphadenopathy, an abrupt change in caliber of PD with distal pancreatic atrophy, grow rate of the cyst of 5 mm or more in 2 years, and a cyst diameter of 30 mm or more are also associated with advanced neoplasia in IPMN. According to the 2018 European guideline, a cyst growth rate of 5 mm or more in 1 year, new onset of diabetes mellitus, and a cyst diameter of 40 mm or more are associated with advanced neoplasia in IPMN. Increased risks of high-grade dysplasia or cancer are also a MPD (main pancreatic duct) between 5 and 9.9 mm, a cystic growth rate

>5 mm/year, serum CA19-9 > 37 U/mL, symptoms, enhancing mural nodules (<5 mm), and/or a cystic diameter >40 mm.

Treatment

When an IPMN at high(er) risk of malignancy is characterized, the treatment of choice is surgery, in surgically fit patients. All guidelines recommend that surgical resection for IPMN should only be performed by experienced surgeons in high-volume centers after consultation by a multidisciplinary team with pancreatic expertise. Standard treatment recommended is pancreatoduodenectomy or left pancreatectomy according to the site and the extent of the disease with lymphadenectomy [10]. Minimally invasive surgery, especially when distal pancreatectomy is indicated, is mostly feasible with good outcome. Most guidelines consider a total pancreatectomy unnecessarily aggressive, especially considering the total endocrine and exocrine insufficiency. For MD-IPMN there is no consensus regarding the best surgical option (total pancreatectomy and partial pancreatectomy followed by close surveillance are possible strategies) [89–93]. In patients with multifocal BD-IPMN, only high-risk BD-IPMN should be resected during surgery, while the other cystic lesions can undergo follow-up. Every cyst should be evaluated individually regarding the presence of sign of degeneration and/or malignancy [13]. The risk of degeneration in multifocal BD-IPMN seems not to be higher compared to the unifocal BD-IPMN (conflicting results can be seen in published literature [14, 94]); therefore a more aggressive approach might be beneficial only in patients with a family history of PDAC [95].

All current guidelines emphasize the importance of intraoperative frozen section. IPMNs originate from pancreatic ducts, both MPD or peripheral ducts; thus the anatomopathological analysis of resection margins and confirmation of disease-free margins are mandatory for radical surgery. This aspect relates very well for those patients with MT-IPMN misdiagnosed as BD-IPMN before surgery, showing involvement of MPD in the pathological examination. When low-grade dysplasia is present in the frozen section, no further resection is required [96]. Obviously, a frozen section will not compensate for potential skip lesions in the MPD [86, 97, 98].

Surveillance After Pancreatectomy

After surgical resection of IPMN, lifelong follow-up and surveillance are recommended because both new IPMN and concomitant PDAC might occur after surgical resection. Resected IPMN-associated cancer should be followed up in the same way as patients with PDAC after pancreatectomy [99].

The main risks of recurrence in patient undergoing surgery for IPMN are HGD (17% of recurrence after surgery [92]) and family history of PDAC (23% of recurrences vs 7% in patients without family history of PDAC [92]). The debate regarding the surgical margins is still open: while Marchegiani et al. [18] found a significantly higher incidence of recurrence in patients with positive margins after surgical resection, He et al. [92] and Kang et al. [100] didn't report any difference in recurrence rate in the positive margins. The risk of recurrence might be correlated not only to other surgical technique but also to the nature of the IPMN and the subtype of the cystic lesion [101–103].

The IAP guideline recommends follow-up at least twice a year for patients with family history of PDAC, surgical resection margin with HGD, and non-intestinal subtype of IPMN. In all other patients with resected IPMN, follow-up every 6–12 months is mandatory. In contrast, the European guideline advises follow-up every 6 months for the first 2 years, followed by yearly surveillance for IPMN with HGD or main duct involvement. All the others should be followed up in the same way as non-resected IPMN.

Recent series underline the increasing risk of recurrence during the surveillance: 4% after 1 year, 25% after 5 years, and 62% after 10 years [92]; the risks of developing a new invasive IPMN are 0%, 8%, and 38% after 1-, 5-, and 10-year follow-up [100]; concomitant PDACs have a cumulative 5- and 10-year incidence of developing of 4.5% and 5.9%, respectively [103]. Therefore, most of the guidelines agree that the surveillance of the patients should not be discontinued if the patient remains fit for surgery.

In some cases, synchronous and metachronous malignancies can be observed during the follow-up of patients with IPMN (20–30% [104]), but the incidence of extra-pancreatic malignancies might be the same with the incidence of cancer in the general population since the percentage of incidence differs from region to region [105].

Surveillance

Follow-up is recommended for all the patients feasible for surgery, without hard indications for resection. Timing of follow-up and the best radiological examination are still a matter of debate. Therefore, the guidelines vary somewhat in their advice.

According to the revised IAP guidelines, an additional EUS is indicated for further inspection of the PCN in patients with clinical or radiological characteristics associated with advanced neoplasia (relative indications for resection) [10]. If on endoscopic ultrasound, hard indications for resection can be ruled out (i.e., enhancing nodule ≥5 mm, PD ≥ 10 mm, cytology suspicious for HGD/invasive cancer), follow-up is advised. The surveillance interval is established on the basis of the main cyst size (Table 2.2): for cyst <1 cm, CT/MRI in 6 months and then every 2 years if there is no change in cyst characteristics and for cyst 1–2 cm, CT/MRI every 6 months for 1 year, then yearly for 2 years, and every 2 years if no change is seen; patients with cyst of 2–3 cm should undergo EUS in 3–6 months and then 1

Table 2.2 Surveillance interval of non-resected PCN stratified by AGA, IAP, and the European guidelines

Guidelines	Cyst type	Cyst size	Surveillance interval	Surveillance modalities
2015 AGA	IPMN	<3 cm	Yearly for 1 year Every 2 years[a]	MRI/MRCP
2017 IAP	IPMN	<1 cm	In 6 months Every 2 years	CT or MRI/MRCP CT or MRI/MRCP
		1–2 cm	Every 6 months for 1 year Yearly for 2 years Every 2 years	CT or MRI/MRCP CT or MRI/MRCP CT or MRI/MRCP
		2–3 cm	3–6 months Yearly	EUS Alternating MRI with EUS
2018 European	IPMN	<4 cm	Every 6 months for 1 year Yearly	CA 19.9, EUS and/or MRI
2018 ACG	IPMN	<1 cm	Every 2 years	MRI
		1–2 cm	Yearly	MRI
		2–3 cm	6–12 months	MRI or EUS

ACG American College of Gastroenterology, *AGA* American Gastroenterological Association, *CA 19.9* cancer antigen 19.9, *CT* computed tomography, *EUS* endoscopic ultrasound, *IAP* International Association of Pancreatology, *IPMN* intraductal papillary mucinous neoplasm, *MRI* magnetic resonance imaging

[a]The 2015 AGA guideline suggests to discontinue the follow-up after 5 years, if there is no change in size or characteristics of the cyst

per year (EUS and MRI can be eventually alternated), and surgery should be considered for young and fit patients who require a prolonged follow-up.

The European guideline [13] recommends follow-up for BD-IPMN < 4 cm without other risk factors with CA19.9 and MRI/MRCP or EUS every 6 months the first year after diagnosis and yearly thereafter.

The best surveillance modality and timing should be evaluated in a large prospective study, possibly within the scope of the PACYFIC study. The PACYFIC study is an international, prospective cohort study aiming to optimize pancreatic cystic neoplasm surveillance (clinical trial number: NTR4505).

During follow-up the 5-year cumulative incidence of developing a concomitant PDAC in patients with IPMN ranges from 2.2% to 8.8% [10]. The follow-up of the patients should be performed with the same radiological technique if possible in order to lower the bias of interobserver measurement of the pancreatic cyst [106].

Conclusions and Recommendations

The detection of pancreatic IPMNs due to the higher rate of radiological examinations and increased life expectancy in the population has led to a global awareness of this entity. Current diagnostic techniques allow to detect and characterize pancreatic cysts, but the natural history of this pathology is still mainly unknown.

Many guidelines have been published and revised in recent years, but the management and surveillance for patients with IPMN remain contradictory.

IPMNs represent a true challenge nowadays, and due to the heterogeneity of these cysts, we truly believe that a multidisciplinary team, and a referred institute, should be mandatory in the decision-making process for these patients. The risk is to underestimate the potential of malignancy of some cystic lesions, leading to a progression of the cyst degeneration with consequent metastasis or invasion of adjacent organs; on the other hand, a too aggressive policy might expose the patients to unnecessary risks of undergoing surgery (morbidity and mortality rates up to 50% and 6.7%, respectively, in high-volume centers) [107] instead of a surveillance program.

Nowadays many questions are still unsolved. For instance, what are the optimal surveillance program and the timing for radiological examination in patients with IPMNs? Which size of BD-IPMN should be considered as indication for surgery and for which size surveillance should not be mandatory? When is better to perform a total pancreatectomy rather than partial pancreatectomy for MD-IPMN?

Further studies and randomized controlled trial are needed to enlighten these aspects since most literature on IPMN is based only on surgical series.

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Chapter 3
Novel Biomarkers of Invasive IPMN

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With the ubiquitous use and high resolution of cross-sectional imaging, pancreatic cystic lesions (PCL) are common incidental findings, detected in up to 20% of abdominal MRI scans in adults [1, 2]. The overall prevalence of PCL in asymptomatic patients undergoing abdominal CT scans is estimated to be 2.6%, with progressive increasing incidence with age, up to 8.7% of patients above 80 [3, 4]. While PCL, especially mucinous PCL, have the capacity to develop into invasive carcinoma, it is increasingly being recognized that their malignant potential is neither uniform nor certain. Indeed, while data from long-term cohorts of PCL have identified a small but ongoing risk to the development of carcinoma [5, 6], this must be balanced against the increasing data demonstrating low yield and high potential morbidity of surgical intervention in elderly patients with non-worrisome PCL [7, 8]. As pancreatic resection remains an effective modality but continues to carry a 1–2% mortality and 30–60% morbidity [9], there remains an unmet need for molecular tools to stratify high-risk/malignant from low-risk lesions.

PCL can be broadly divided into nonmucinous and mucinous lesions, with mucinous lesions (intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasm (MCN)), accounting for 10–50% and harboring malignant potential [10–12]. Even within these mucinous lesions, in surgical resection cohorts, main-duct and mixed-type IPMNs have a 48% or 42% chance of harboring invasive carcinoma in comparison to branch-duct lesions (BD-IPMN) with only an 11% chance [13, 14]. While imaging characteristics, including cyst size (typically greater than 3 or 4 cm), main pancreatic duct dilation (typically greater than 5 mm or 10 mm), and the presence of solid components or mural nodules, may predict the presence of high-grade dysplasia (HGD) or invasive disease on surgical resection, all of these

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clinical features alone have poor overall accuracy in predicting this important outcome [15–18]. Overall, morphology on endoscopic ultrasound (EUS) is not accurate in distinguishing nonmucinous from mucinous cysts without fluid analysis, let alone assessing dysplastic grade [19]. As discussed in detail in the previous chapter, evidence-based criteria and clinical guidelines have been developed to aid in the appropriate surveillance and management of PCL [16–18, 20]. Despite widespread clinical adoption, these guidelines are imperfect, as the clinical/imaging features they rely upon are themselves imperfect [21, 22]. As a whole, earlier iterations of the guidelines like the Sendai criteria while highly sensitive (97–100%) were not specific (20–30%) for BD-IPMN harboring advanced neoplasia, and while subsequent guidelines like the Fukuoka and AGA guidelines improve the specificity (34.5–45.6%), it was at the cost of reduced sensitivity (7.3–35.2%) [23–25]. Furthermore, these guidelines rely on static assessments of clinical features and do not take into account cyst biology, which may be highly variable.

Ultimately, the objective of any surveillance program is to identify lesions at a preinvasive state or, at most, with HGD/carcinoma in situ (CIS), when curative therapy can be rendered. In the case of pancreatic ductal adenocarcinoma (PDAC), precursor lesions are generally considered to be IPMN or pancreatic intraepithelial neoplasia (PanIN). PanIN are classified into three grades: PanIN-1 and PanIN-2, which are low-grade lesions, and PanIN-3 demonstrating pronounced cytologic and architectural atypia, equivalent to in situ carcinoma [26]. However, given their microscopic nature and heterogeneous distribution, PanIN-3 are not reliably imaged on cross-sectional or ultrasonographic imaging. For example, in a cohort ($n = 125$) of patients who are at high risk for pancreatic cancer due to strong family histories, among those patients who underwent surgical resection for multifocal IPMN ($n = 5$), the location of the most dysplastic histologic lesions (PanIN-3) did not correlate with the preoperatively visualized lesion [27]. This illustrates both the limitations of imaging for resolving microcellular changes and the "field defect" that is apparent in patients with high-risk pancreatic lesions. That said, IPMNs are the only clinically readily appreciated precursor lesions to PDAC [28], and thus the segregation of IPMNs that are at high risk for transformation represents an opportunity for resection before the development of invasive cancer with improved overall survival [29, 30]. However, the last 20 years of clinical experience has continued to demonstrate that the resection of cysts with low risk of malignant transformation carries significant up-front surgical morbidity and mortality, even in high-volume centers, without significant long-term benefit [31, 32].

PCL are readily imaged with ultrasound, CT, and MRI and are relatively safely sampled utilizing EUS-guided FNA with cyst aspiration. The current standard approach is for cyst fluid to be submitted for cytologic analysis and biochemical analysis for carcinoembryonic antigen (CEA) and amylase. Overall, FNA is safe, but the diagnostic yield with currently available clinical testing is limited with poor sensitivity, but high specificity (sensitivity 27–48%, specificity 83–100%) [33–35]. As such, there has been varying enthusiasm for EUS FNA, driven by the dearth of biomarkers and inadequacy of cytology to reliably assess risk in these PCL [33]. The hope of many researchers, including ourselves, is that a new generation of

biomarkers together in concert with clinical parameters may influence future guidelines and establish cyst fluid analysis as critical for clinical risk stratification of PCL.

In this chapter, we will review the published data on biomarkers at varying stages of investigation used to risk stratify IPMN and detect invasive carcinoma arising from IPMN. We will first outline the data for routinely used, clinically available fluid studies (Table 3.1) and review the data on novel DNA-, RNA-, and protein-based cyst fluid biomarkers and biomarkers from other sources (circulating cells, pancreatic juice, etc.).

Biomarkers in Current Clinical Practice for the Evaluation of PCL

Amylase

Cyst fluid amylase indicates a connection between the cyst and the ductal system with high specificity [11]. However, the level cannot differentiate between pseudocyst, IPMN, or MCN [36]. In one study, cyst fluid amylase levels decreased with increasing levels of dysplasia in MCN, but these levels were not significantly different [37]. As such, amylase cannot be used to adequately rule out malignancy or assess risk of malignant transformation. Interestingly, in one study, serum levels of amylase were significantly lower in patients with surgically resected invasive IPMN in comparison to matched controls (OR 9.6, 2.99–35.1) [38].

CEA

CEA is a glycoprotein on the cell surface of mucin-producing epithelium. CEA is primarily useful for differentiating mucinous from nonmucinous PCL [11]. Various cutoff values have been explored for CEA in pancreatic cyst fluid with varying sensitivity and specificity. Using the original, traditional cutoff of 192 ng/ml, the area under the curve for differentiating mucinous vs nonmucinous cysts was 0.79 with a likelihood ratio of 4.37 [19, 35]. However, subsequent studies have utilized a cutoff of 30.7 ng/mL (sensitivity 88.3, specificity 77.8%) or 105 ng/mL (sensitivity 70%, specificity 63%) [39, 40]. CEA may be best used for predicting a nonmucinous PCL, as a level below 5 ng/ml effectively rules out a mucinous tumor with a positive predictive value of 94% [41]. Importantly, CEA cannot differentiate IPMN from other mucin-producing cyst types (i.e., MCN).

Beyond differentiating cyst type, studies have evaluated using CEA to detect malignant transformation and dysplasia. While early studies using a cyst CEA cutoff of 200 ng/ml resulted in a sensitivity of 90% and specificity of 72% in identifying HGD or invasive carcinoma on surgical pathology in IPMN [42], subsequent

Table 3.1 Biomarkers in current clinical practice for PCL risk stratification

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
Cyst amylase	Scourtas et al. [37]	2017	136 MCN	Surgical pathology from resection			Mean amylase lower for invasive MCN than MCN with intermediate-grade dysplasia, which was lower than MCN with low-grade dysplasia
Serum amylase	Yagi et al. [38]	2016	142 surgically resected IPMN	Surgical pathology – invasive IPMN			Low serum amylase (<16 IU/L) associated with higher risk of invasive IPMN. OR 9.6 (2.99–35.1)
Cyst CEA	Brugge et al. [19]	2005	112	Mucinous vs nonmucinous	75%	83.6%	CEA > 192 ng/ml Accuracy: 79%
Cyst CEA	Maire et al. [42]	2008	41	IPMN with HGD or invasive carcinoma	90%	71%	CEA ≥ 200 ng/ml PPV 50%, NPV 96%
Cyst CEA	Correa-Gallego et al. [43]	2009	197	IPMN with carcinoma in situ or invasive carcinoma	47%	40%	CEA ≥ 200 ng/ml PPV 20%, NPV 70%
Cyst CEA	Kucera et al. [44]	2012	47	IPMN with HGD or invasive carcinoma	52.4%	42.3%	CEA ≥ 200 ng/ml PPV 42.3%, NPV 52.4%
Cyst CEA	Thornton et al. [35]	2013	1438	Mucinous vs nonmucinous	63%	88%	Meta-analysis
Cyst CEA	Ngamruengphong et al. [45]	2013	504	All malignant pancreatic cystic lesions (HGD or invasive cancer)	63% 65% for mucinous only	63% 66% for mucinous only	Meta-analysis of studies of pancreatic cysts
Cyst CEA	Gaddam et al. [39]	2015	226	Mucinous vs nonmucinous	105 ng/ml: 70% 192 ng/ml: 61%	105 ng/ml: 63% 192 ng/ml: 77%	Tested various cutoffs

Cyst CEA	Jin et al. [40]	2015	86	Mucinous vs nonmucinous	88.3%	77.8%	CEA > 30.7 ng/ml
Serum CEA	Fritz et al. [49]	2011	142	Surgically resected IPMN – invasive vs noninvasive	40%	92.4%	CEA of 5 ug/l PPV 74.1%, NPV 73.9%
Cyst Ca19-9	Pais et al. [51]	2007	74	Malignant IPMN on surgical pathology	60%	75%	Ca19-9 of 10,000 U/ml PPV 75% NPV 60% Mean Ca19-9 not different between malignant and benign cysts
Serum Ca19-9	Fritz et al. [49]	2011	142	Surgically resected IPMN – invasive vs noninvasive	74%	85.9%	CA19-9 of 37 u/ml PPV 74% NPV 85.9%
Serum Ca19-9	Kim et al. [52]	2015	367	HGD and invasive IPMN on surgical pathology	34.2%	92.4%	"Elevated Ca19-9"
Serum Ca19-9	Wang et al. [53]	2015	1629	Malignant or invasive IPMN	Malignant: 40% Invasive: 52%	Malignant: 89% Invasive: 88%	Ca19-9 35 ng/ml Meta-analysis
Cyst cytology	Sedlack et al. [64]	2002	111	Malignant/potentially malignant vs benign cysts	27%	100%	Accuracy: 55%
Cyst cytology	Brugge et al. [19]	2005	112	Mucinous vs nonmucinous	34.5%	83.3%	Accuracy: 58.7%
Cyst cytology – atypical epithelial cells	Pitman et al. [59]	2008	20	Invasive or malignant IPMN	Malignant: 83% Invasive: 88%%	Malignant: 67%, Invasive: 59%	Addition of CEA > 2500 ng/ml improves sensitivity, but decreases specificity
Cyst cytology	Morris-Stiff et al. [58]	2010	121	Mucinous vs nonmucinous	38%	90%	PPV: 90% NPV: 31%

(continued)

Table 3.1 (continued)

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
Cyst cytology	Cizinger et al. [56]	2011	198	Mucinous vs nonmucinous Subgroup with malignant pathology	Mucinous: 43% Malignant: 37.5%	Mucinous: 96.2%, Malignant: 96%	Mucinous accuracy: 58%, Malignant accuracy: 74.7%
Cyst cytology	de Jong et al. [55]	2012	32	Mucinous vs nonmucinous	48%	100%	
Cytopathology KRAS GNAS	Bournet [79]	2016	37	HGD and invasive carcinoma	Cytology: 55% KRAS: 66% GNAS: 19% Cytology+ KRAS: 92% Cytology+ GNAS: 62% Cytology+ KRAS+ GNAS 92%	Cytology: 100%, KRAS: 50%, GNAS: 70%, Cytology+ KRAS: 50%, Cytology+ GNAS: 70%, Cytology+ KRAS+ GNAS: 50%	
Cyst cytology	Tanaka et al. [65]	2019	743	IPMN with HGD, CIS, invasive carcinoma	57%	84%	Systematic review
Pancreatic juice cytology	Tanaka et al. [65]	2019	537	IPMN with HGD, CIS, invasive carcinoma	54%	91%	Systematic review
Pancreatic juice cytology	Kawada et al. [165]	2016	50	Malignant IPMN without mural nodule	94% without mural nodule vs 53% with mural nodule		

studies in mucinous cystic neoplasm and IPMN have shown that CEA levels in benign and malignant cysts overlap significantly, with poor test characteristics [37, 43–45]. This result has been demonstrated in multiple studies, suggesting that CEA alone should not be used to detect invasive PCL or IPMN [19, 41, 46, 47]. Serum CEA is elevated in 60% of patients with pancreatic ductal adenocarcinoma. While serum CEA has a high specificity for invasive IPMN, the sensitivity of serum CEA for malignant and invasive IPMN is only 18% [48, 49].

CA19-9

Carbohydrate antigen 19-9 (CA19-9) is a tumor-associated glycoprotein that is elevated in the serum of 85% of patients with PDAC [48]. However about 5–10% of the population is unable to produce Ca19-9 due to lack of an enzyme needed for epitope production, which does limit its use as biomarker [50]. In cyst fluid, Ca19-9 performs worse than CEA for differentiating mucinous from nonmucinous cysts [19]. Cyst fluid Ca19-9 is also not useful in differentiating malignant from benign IPMN with one study showing no difference in levels between these groups [51]. Serum Ca19-9 may be useful in ruling in invasive IPMN with reasonable specificities and poor sensitivities [49, 52]. In a meta-analysis, the sensitivity and specificity of Ca19-9 level of 35 ng/ml measured in serum were 52% and 88%, respectively, for detecting invasive IPMN [53].

Cytology

While routinely performed, standard cytological evaluation of cyst fluid is hampered by low cellular content, the focal nature of dysplasia, and high interobserver variability [54]. The overall performance of cytology in PCL diagnosis ranges with low sensitivities of 27–48% and high specificities of 83–100% [19, 33, 55–58]. The pooled sensitivity and specificity in a systematic review and meta-analysis were 54% and 93%, respectively, in differentiating mucinous from nonmucinous cysts [35]. To improve the clinical utility of cyst fluid cytology, the inclusion of atypical epithelial cells has been suggested, and this improved the sensitivity and specificity to 72% and 85%, respectively, for identifying malignant cysts [59, 60]. However, the clinical utility of this system is limited as interobserver agreement is poor without significant experience [61, 62]. Yield may also be increased by cyst wall biopsy or other technical maneuvers, but there remains a significant need for improvement [36, 63, 64]. Cytologic analysis of pancreatic juice collected during ERCP in patients with IPMN has similar test characteristic to EUS FNA acquired cyst fluid cytology with recent systematic review showing a sensitivity of 54% and specificity of 91% [65].

Overall, with a low diagnostic accuracy of 8–59%, cytology from FNA while highly predictive if positive has too poor a negative predictive value to exclude advanced neoplastic change in a PCL [19, 33, 56, 64].

DNA Testing

DNA testing has emerged as a potential adjunct to risk stratifying IPMN in part as DNA from lysed or exfoliated epithelium from cyst lining is abundant and can be analyzed for genetic mutations [66–68]. In addition to differentiating mucinous from nonmucinous cysts, it has also been proposed for use in detecting advanced neoplasia in IPMN as we review below (Table 3.2).

KRAS

Mutations in KRAS, an oncogene that encodes a membrane-bound guanosine triphosphate (GTP) binding protein upstream of MAPK signaling pathways [69], are present in more than 90% of pancreatic adenocarcinomas. Furthermore, successive accumulation of alterations in cancer-associated genes including *KRAS*, *p16/CDKN2A*, *TP53*, and *SMAD4/DPC4* has been noted in the progression from PanIN-1/2 lesions to high-grade PanIN-3 lesions [70]. Given the ubiquity and critical nature of KRAS in pancreatic cancer, its early detection has been seen as a promising target for risk stratification of lesions that have undergone malignant transformation in PCL [71, 72].

Khalid et al. first examined the potential role of evaluating Kras mutations in cyst fluid in a small cohort that included 11 malignant lesions [66]. This was followed by a series of additional studies that studied the presence of KRAS mutations as well as other DNA parameters (quantity, quality, etc.) which showed that the presence of KRAS mutation was specific (80–100%) for mucinous cysts but not sensitive (33–86%) [73–79]. In addition, the majority of these studies did not find KRAS to have significant accuracy in differentiating malignant from premalignant mucinous cysts alone, though they suggested combinations with other parameters like allelic loss to improve sensitivity/specificity (sensitivity of 54% and specificity of 46%) [65]. Of particular note is the multicenter PANDA study, in which the presence of KRAS mutations in pancreatic cyst fluid was useful in differentiating mucinous cysts from nonmucinous cysts with a specificity of 96% but a low sensitivity of 45% [68]. The presence of a Kras mutation along with an allelic loss was only 37% sensitive and 96% specific for a malignant lesion [68]. In a real-world follow-up study of prospectively sampled patients utilizing this commercially available technique, KRAS mutation presence had a 42% sensitivity and 90% specificity for mucinous lesion, but did not have adequate accuracy for high-risk lesions [80]. Overall, the test characteristics of KRAS vary on the setting, definition, and design (Table 3.1).

Table 3.2 Novel DNA-based biomarkers

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
KRAS	Khalid et al. [66]	2005	36	Malignant pathology	91% 91% when followed by allelic loss	86% 93% when followed by allelic loss	MCN
KRAS and LOH	Schoedel et al. [73]	2006	16	HGD/cancer vs LGD/MGD			KRAS plus LOH in 50% HGD/cancer vs 8% in LGD/MGD
KRAS and LOH	Schoedel et al. [73]	2006	16	Carcinoma vs adenoma or borderline lPMN			50% of carcinomas and 8% of others
KRAS, allelic loss, mutational amplitude, mean number of mutations	Khalid et al. [68]	2009	113	Mucinous vs nonmucinous IPMN and MCN	KRAS: 45% KRAS and allelic loss: 19% MALA: 67% Mean number of mutations: 62%	KRAS: 96% KRAS and allelic loss: 100% MALA: 66% Mean number of mutations: 62%	
KRAS, allelic loss, mutational amplitude, DNA quality and quantity	Khalid et al. [68]	2009	113	Malignant vs premalignant IPMN and MCN	KRAS and allelic loss: 37% MALA: 90% DNA quantity: 75% Mean DNA quality: 75%	KRAS and allelic loss: 96% MALA: 67% DNA quantity: 79% Mean DNA quality: 67%	
CEA, molecular analysis, KRAS, allelic imbalance, DNA concentration	Sawney et al. [74]	2009	100	Mucinous vs nonmucinous	CEA: 82% Molecular analysis: 77 KRAS: 11% Allelic imbalance: 70% DNA concentration: 29%	CEA: 100% Molecular analysis: 100% KRAS: 100% Allelic imbalance: 100% DNA concentration: 100%	

(continued)

Table 3.2 (continued)

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
PathFinder TG KRAS, LOH, DNA quantity/quality	Shen et al. [75]	2009	35	CIS and invasive cancer All cysts	83%	100%	PPV 100%
KRAS	Nikiforova et al. [78]	2013	618	Mucinous vs nonmucinous	54%	100%	
KRAS and GNAS	Siddiqui et al. [95]	2013	25	IPMN vs other cysts	KRAS: 67% GNAS: 44% Either: 100%	KRAS: 69% GNAS: 100% Either: 69%	
KRAS	Al-Haddad et al. [80]	2014	48 surgically resected cysts 38 mucinous cysts 35 IPMN	Mucinous vs nonmucinous	42.1%	90%	
KRAS and GNAS	Singhi et al. [96]	2014	91	IPMN vs other cysts	KRAS: 70% GNAS: 36% Either: 84%	KRAS: 98% GNAS: 100% Either: 98%	
KRAS, elevated DNA, ≥2 LOH, and KRAS and ≥2 LOH	Winner et al. [87]	2015	40	Invasive adenocarcinoma vs benign	KRAS: 44.4% Elevated DNA: 66.7% ≥2 LOH: 62.5% KRAS and ≥LOH: 50%	KRAS: 55.6% Elevated DNA: 77.8% ≥2 LOH: 76.9% KRAS and ≥LOH: 96.2%	Retrospective
KRAS and LOH	Guo et al. [89]	2016	428	Mucinous vs nonmucinous Benign vs malignant cysts	Mucinous vs nonmucinous KRAS: 47%, LOH: 63%; Benign vs malignant KRAS: 59% LOH: 89%	Mucinous vs nonmucinous KRAS: 98% LOH: 76% Benign vs malignant KRAS: 78% LOH: 69%	Systematic review and meta-analysis

NGS: KRAS/GNAS, *TP53/PIK3CA/PTEN*	Singhi et al. [97]	2018	595 – NGS 159 – KRAS/ GNAS	HGD or invasive	89%	100%	
KRAS	Tanaka et al. [65]	2019	333	HGD, CIS, invasive carcinoma	54%	46%	Systematic review
GNAS	Tanaka et al. [65]	2019	144	HGD, CIS, invasive carcinoma	29%	46%	Systematic review
Telomerase	Hata et al. [101]	2016	119 cysts; 74 IPMN	HGD or invasive cancer	74.2% in all 74.2% in IPMN	93.2% all 86.1% in IPMN	Cystic lesions
TP53	Tanaka et al. [65]	2019	780	HGD, CIS, invasive carcinoma	31%	93%	TP53 from surgical pathology
SMAD4	Tanaka et al. [65]	2019	291	HGD, CIS, invasive carcinoma	14%	99%	
P16	Tanaka et al. [65]	2019	325	HGD, CIS, invasive carcinoma	52%	46%	
Cell-free DNA	Berger et al. [163]	2016	21 IPMN, 38 controls, 24 metastatic PDAC, 26 SCA, 16 borderline IPMN	PDAC vs IPMN vs control	IPMN vs control: 81% PDAC vs control: 83% PDAC vs IPMN: 75%	IPMN vs control: 84% 92% PDAC vs control: 94 PDAC vs IPMN: 71%	

There may be a variety of reasons why KRAS mutations have not proven to be an accurate biomarker for high-risk lesions. KRAS mutations are seen in a significant number of patients with chronic pancreatitis who do not go on to develop pancreatic cancer but have a high prevalence of PanIN lesions [81, 82]. KRAS mutations do not differ by degree of dysplasia as this may be an earlier event in carcinogenesis [83, 84]. In addition, in an autopsy series of 138 patients who died of non-pancreatic-related causes, 38 patients were found with PanIN lesions and 12 were found with oncogenic Kras mutations, suggesting that these mutations may occur outside of the context of clinically significant carcinogenesis [85]. Alternatively, there may be differential presence of the mutations varying by epithelial subtype of IPMN. In one study, KRAS mutations were more common in gastric- and pancreatobiliary-type IPMN than in intestinal or oncocytic subtypes and were more common in tubular and minimally invasive carcinoma than in those with mucinous or oncocytic carcinoma when surgical specimens were stained [86].

DNA Quantity, Loss of Heterozygosity, and Mean Allelic Loss Amplitude (MALA)

DNA quantity can be assessed as a part of molecular analysis of cyst fluid and was routinely included in the abovementioned studies examining KRAS gene mutations in cyst fluid. In one study, the sensitivity and specificity of a DNA level >40 ng/μl for differentiating mucinous from nonmucinous cysts were 29% and 100%, respectively [74]. In two other studies, an elevated DNA level as confirmed by an optical density >10 had sensitivities of 75% and 67% and specificities of 79% and 77.8% for differentiating malignant from premalignant cysts [68, 87]. Overall however, DNA quantity alone, essentially as a surrogate for cellularity in the cyst fluid, has not been felt to be a highly accurate biomarker for high-risk lesions.

Loss of heterozygosity (LOH), the loss of one copy of a tumor suppressor gene, is evaluated with a panel of microsatellite markers [88]. In a meta-analysis, the sensitivity and specificity of LOH in cyst fluid for distinguishing mucinous cysts from nonmucinous cysts were 63% and 76%, respectively [89]. For differentiating malignant from benign cysts, LOH yielded a sensitivity of 89% and specificity of 69% [89]. When KRAS and LOH were combined, the sensitivity for differentiating malignant from benign cysts was 50%, with a specificity of 96% [87]. In another study, the combination of KRAS mutations and LOH was present in 50% of IPMN with high-grade dysplasia and cancer versus 8% of IPMN with low-grade or indeterminate dysplasia [73].

The mean allelic loss amplitude (MALA) is another metric of DNA analysis that has been shown to have a sensitivity and specificity of 90% and 67%, respectively, for detecting malignant cysts in one study [68]. When KRAS was combined with MALA, the specificity for detecting malignant cysts was improved to 96%, but the sensitivity was only 37% [68].

As each of these assessments proved promising but insufficient, investigators have suggested the combination of KRAS gene mutations, DNA concentration, and allelic loss in cyst fluid analysis together. A commercialized version of these assays that combines KRAS analysis, LOH, and DNA quantity for cyst fluid analysis (PathFinder TG, Interpace Diagnostics) demonstrated a sensitivity and specificity of 83% and 100%, respectively, in a single study [75]. A retrospective review of 492 patients utilizing this molecular diagnostic testing, integrated with first-line test results (cytology, fluid chemistry, and imaging), suggested a method called integrated molecular pathology (IMP) and compared the results to real-world decisions and guidelines recommendations [90, 91]. Among the cohort of patients (n = 209) with surgical pathology available, IMP demonstrated a sensitivity and specificity of 81.0% and 78.0%, respectively, which was significantly better than the Sendai guidelines.

GNAS

GNAS is an oncogene that encodes the alpha subunit of stimulatory G protein (Gs-α) that is ubiquitously expressed for G-protein stimulatory signaling, especially in several hormonal axes. Germline mutations are associated with McCune-Albright syndrome, and somatic mutations are seen in a variety of endocrine tumors (Leydig tumors, ovarian granular cell tumors, pituitary tumors) [92–94]. Mutations at codon 201 have been shown to be useful in distinguishing IPMN from other pancreatic cyst types, with studies showing that GNAS mutations occur in 44–61% of IPMN, while there were no mutations in other mucinous cysts [83, 95]. In the initial work examining the association of GNAS with cystic lesions of the pancreas, Wu et al. examined 19 IPMN sequenced for 169 genes utilizing massively parallel sequencing to identify even very small cell populations. In this cohort, ~81% had a KRAS mutation (G12D, G12V, or G12R) and ~66% had a GNAS mutation (R201H or R201C) with no expression in serous cyst adenoma and no correlation to grade, size, or prognosis [94]. In a large prospective study on cyst fluid preoperatively, the presence of a mutation in either KRAS or GNAS had a sensitivity of 84% with a specificity of 98% for detecting IPMN [96]. Using next-generation sequencing, KRAS and/or GNAS mutations were present in all IPMN when confirmed by surgical pathology [97]. However, utilizing 86 surgically resected patient specimens, GNAS mutations were found to be more frequently associated with intestinal-type (100%) and pancreatobiliary-type (71%) IPMN as opposed to gastric (51%) and oncocytic (0%) IPMN, but there was no association with location, malignancy, or survival [98]. In a study from 291 pancreatic juice aspirates from high-risk patients, there was no association with GNAS mutations and prognosis or histologic grade of cystic lesions. In fact, the presence of the mutation predicted the subsequent radiologic development of cysts in a small group of patients [99]. In a recent systematic

review, the sensitivity and specificity for GNAS alone in differentiating HGD, CIS, and invasive carcinoma from benign cysts were 29% and 46%, respectively [65, 79].

When combined with KRAS testing, the sensitivity and specificity in diagnosing IPMN when either marker was positive in one small pilot study were 100% and 69%, respectively [95]. This finding is supported by mouse models suggesting the cooperation of KRAS and GNAS to promote pancreatic tumorigenesis [100].

Telomerase

Telomerase is a ribonucleoprotein that regulates telomere length which is critical in stem cells and most cancer cells. Telomerase and telomere length has been shown to be associated with IPMN progression, as it is in many cancers [46]. In a study of cyst fluid from 219 patients, telomerase activity was higher in cysts with HGD or invasive cancer and was an independent predictor of high-grade dysplasia or invasive cancer on surgical resection [101]. The sensitivity and specificity for detecting advanced neoplasia in IPMN were 74.2% and 86.1%, respectively [101].

Tumor Suppressor Genes

Loss of heterozygosity of TP53 was seen only in invasive IPMN in one study of 23 patients with surgically resected IPMN [102]. Another tumor suppressor, p16 or cyclin-dependent kinase inhibitor 2a (CDKN2A), may be useful for discriminating IPMN with low to intermediate dysplasia from IPMN with carcinoma [48, 103]. p16 is more commonly inactivated in IPMN with carcinoma than borderline IPMN [104]. In combination with p16 inactivation, inactivation of TP53 is found in 20% of low-grade tumors, 33% of noninvasive carcinomas, and in all invasive carcinomas in IPMN [48, 102]. In another retrospective study of 172 IPMNs, TP53 mutation overexpression was associated with poorer survival in IPMN and worse histologic grade [86]. Overall, estimates of the prevalence of Tp53 and p16 in IPMN vary, and these mutations may occur early in malignant transformation limiting the use of these genes as biomarkers for invasive IPMN [46]. In a recent systematic review, TP53 in 780 IPMNs yielded a sensitivity of 31% and specificity of 93% in differentiating HGD, CIS, and invasive carcinoma from benign IPMN [65]. However, a recent study looking at the combination of mutations in TP53, PIK3CA, and/or PTEN along with KRAS and/or GNAS mutations demonstrated a sensitivity of 89% and specificity of 100% for detecting advanced neoplasia in IPMN [97].

Other Genes

Inactivating mutations in SMAD4 occur late in the progression of precursor lesions to pancreatic cancer, likely working as a critical mediator between the extracellular matrix and the TGF-ß family as a well as a substrate of Erk/MAPK and GSK3 [28]. Mouse models have demonstrated that haploinsufficiency of SMAD4 in association with oncogenic KRAS is associated with macroscopic, mucinous cystic lesions in the body/tail of the pancreas consistent with human MCN [105]. In a retrospective study of 172 IPMNs, SMAD4 loss was associated with worse histologic grade, invasive phenotypes, and worse survival [86]. In the systematic review by Tanaka et al., surgical pathology stained for SMAD4 in 291 IPMNs yielded a sensitivity of 14% and specificity of 99% in differentiating HGD, CIS, and invasive carcinoma from benign IPMN [65].

Brahma-related gene 1 (BRG1) is a member of the SWI/SNF family of proteins with helicase and ATPase activity that can regulate transcription by altering chromatin superstructure [106]. Mutations are common in lung cancer cell lines, medulloblastoma, AML, and pancreatic cancer. In mouse models, acinar-specific deletion of BRG1, in conjunction with oncogenic KRAS mutations, leads to the development of cystic neoplastic lesions reminiscent of MCN vs IPMN in humans [107–109]. In one study, BRG1 was found to be inactivated in 53.3% of IPMN, and mutation was more common in high-grade IPMN than in intermediate- and low-grade IPMN [110]. The role of BRG1 in human IPMN-associated carcinogenesis continues to be investigated.

Next-Generation Sequencing (NGS)/Molecular Panels

NGS allows for high-throughput sequencing that is more sensitive than Sanger sequencing, facilitating rapid detection of mutations even at very low frequencies [97, 111]. In an early study of NGS of cyst fluid using a number of genes previously implicated in tumorigenesis in pancreatic neoplasia, NGS was most valuable in identifying mucinous cysts that were thought to be nonmucinous by CEA level [81]. A study evaluating cyst fluid from IPMN for 51 cancer-associated genes found adequate DNA for analysis in 70% of cysts [112]. GNAS and/or KRAS were present in 92% of IPMN, and TP53, BRAF, and p16 mutations were observed more frequently in high-grade IPMN or IPMN-associated carcinomas [112]. Subsequently, in a recent study utilizing a cohort of 102 patients where diagnostic pathology was available, an NGS panel consisting of mutations in TP53, PIK3CA, and/or PTEN with KRAS and/or GNAS mutations had a sensitivity of 89% and specificity of 100% for detecting advanced neoplasia in IPMN using cyst fluid preoperatively [97]. While panel testing is attractive due to the identification of small populations of cells with mutations and overcoming limitations of individual gene analysis, this has to be balanced by the considerable cost as well as complexity in the completion and analysis of this kind of testing. Given these initial promising results, these panels are undergoing prospective validation to establish their utility and assess their precise role in guiding clinical decision-making.

Epigenetic Alterations

There has also been research into DNA methylation as an epigenetic factor related to tumorigenesis for use as a biomarker in IPMN, but this has not been extensively studied for use in pancreatic cyst fluid. Promoter hypermethylation at cytosine-phosphoate-guanine (CpG) islands leads to tumor suppressor gene silencing [48]. In one study of 51 IPMNs, >80% of IPMN exhibited hypermethylation of at least one of seven CpG islands [113]. ppENK and p16 hypermethylation were more common in high-grade IPMN than in low-grade IPMN, and the average number of methylated loci was higher in high-grade than low-grade IPMN [113]. In another study, high-grade IPMNs had a higher number of hypermethylated genes than low-grade IPMNs [114]. The genes BNIP3, PTCHD2, SOX17, NXPH1, and EBF3 were more likely to be hypermethylated in IPMN with high-grade dysplasia than with low-grade dysplasia or normal tissue [114]. Studies testing serum-based cell-free DNA promoter hypermethylation as a marker of pancreatic cancer have been limited by small power, and no studies have been performed using this in IPMN [115].

RNA-Based Biomarkers

MicroRNAs (miRNAs) are small, noncoding RNA molecules that are involved in epigenetic posttranscriptional gene regulation [116]. Variation in miRNA has been shown to be involved in tumorigenesis and progression in pancreatic ductal adenocarcinoma [117]. miRNA-21 has been found to be clinically useful in IPMN differentiation in a few small studies. In cyst fluid, miRNA-21 was able to differentiate mucinous from nonmucinous cysts with a sensitivity of 80% and specificity of 76% [118]. In another study, there was a gradient of increasing miRNA-21 and miRNA-221 expression from benign to premalignant to malignant cysts [119]. Expression of miRNA-155 and miRNA-21 were significantly higher in invasive IPMN compared to noninvasive IPMN in surgically resected lesions, while miRNA-101 was higher in noninvasive IPMN compared to invasive IPMN [120]. miRNA-216 and miRNA-217 expression increased in a gradient from low-grade IPMN to high-grade IPMN to invasive cancer [121]. Finally, in a study of 65 cyst fluid samples, a panel of 18 miRNAs separated high-grade from low-grade IPMN, and a model using a panel of 9 miRNAs could predict cyst pathology improving surgical management versus conservative management with a sensitivity of 89% and a specificity of 100% [122]. Research into the clinical utility of miRNA is still evolving and has been primarily exploratory in small studies, but results are promising (Table 3.3).

Protein-Based Biomarkers

Several protein-based biomarkers as well as large-scale proteomic analyses have been conducted in attempts to identify possible biomarkers for invasive IPMN and are summarized in Table 3.4.

Table 3.3 Novel RNA-based biomarkers

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
miRNA	Rye et al. [118]	2011	40	Mucinous vs nonmucinous	76%	80%	miRNA 21
miRNA	Matthaei et al. [122]	2012	120	Surgical resection vs conservative management	89%	100%	9 miRNA predicted need for surgical resection (high-grade IPMN, PanNETS and SPNs) vs conservative management (low-grade IPMN, SCA)
miRNA	Farrell et al. [119]	2013	38	Malignant vs premalignant vs benign			miR-221 higher in malignant vs benign cysts miR-21 higher in malignant than premalignant than benign
miRNA	Caponi et al. [120]	2013	86	Invasive vs noninvasive IPMN vs benign cyst			miR-21 and miR-155 were higher in invasive than noninvasive IPMN, miR-101 was higher in noninvasive and benign IPMN than invasive IPMN. Higher miR-21 predicted worse overall survival and independently predicted mortality and disease progression
miRNA	Wang et al. [121]	2015	17	High grade/invasive vs low grade/benign			miR-216 and miR-217 levels increased from low to high grade to cancer

Table 3.4 Novel Protein-Based Biomarkers

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
SHH	Tanaka et al. [65]	2019	148	HGD, CIS, invasive carcinoma	81%	66%	SHH from surgical pathology
Proteomics	Corcos et al. [125]	2012	43	Low- and moderate-grade dysplasia vs IPMN with severe dysplasia or adenocarcinoma			31 peaks were expressed differentially in IPMN with low to moderate dysplasia versus IPMN with severe dysplasia or invasive adenocarcinoma. 5 unspecified proteins were accurate in differentiating the groups with an AUC of 0.88 on receiver operator curve (ROC) analysis
MUC1 from pancreatic juice from ERCP	Shimamoto et al. [134]	2010	34	IPMN with carcinoma vs IPMN adenoma	88.9%	71.4%	PPV 83.3%, NPV 81.3%, pancreatic juice from ERCP
MUC	Maker et al. [129]	2011	40	LGD IPMN vs HGN/ invasive cancer			MUC2 and MUC4 concentrations were higher in cyst fluid in patients with IPMN with high-grade dysplasia or carcinoma compared to IPMN with low- to moderate-grade dysplasia. Serum MUC5 AC was higher in patients with IPMN with high-grade dysplasia
MUC5AC	Cao et al. [127]	2013	44 prevalidation 22 blinded	Mucinous vs nonmucinous	87–89%	100%	
MUC1, MUC2, MUC5 AC, and MUC6	Sai et al. [133]	2013	44	Malignant vs benign branch-duct IPMN	92%	100%	100% PPV, 97% NPV; pancreatic duct lavage cytology from ERCP
MUC1	Jabbar et al. [131]	2014	29	Malignant vs nonmalignant	87.5%	92.3%	89.7% accurate, 92.3% PPV, 85.7% NPV

MUC 5 AC and MUC2	Jabbar et al. [130]	2018	68	Malignant/ premalignant vs benign			97% accurate in identifying malignant from premalignant lesions
Plec-1	Bausch et al. [147]	2009	37	HGD and invasive carcinoma vs low and moderate dysplasia	84%	83%	
Amphiregulin	Tun et al. [149]	2012	33	HGD/cancer in IPMN or MCN	83%	73%	
Spink 1	Raty et al. [150]	2013	61	Main/mixed-duct IPMN and MCn vs side-branch IPMN and SCA	85%	84%	@ 118 μg/l
Das 1	Das et al. [139]	2014	38	High-risk IPMN = Intestinal IPN with intermediate-grade dysplasia, IPMN-gastric, intestinal, pancreaticobiliary, or oncocytic with HGD, and invasive IPMN	89%	100%	
PGE_2	Yip-Schneider et al. [145]	2017	100	High-grade dysplasia and invasive carcinoma	PGE_2 1.1 pg/μl: 63% CEA > 192 ng/ml and PGE_2 0.5 pg/μl: 78%	PGE_2 1.1 pg/μl: 79% CEA > 192 ng/ml and PGE_2 0.5 pg/μl: 100%	Mean cyst fluid levels higher in HGD/ invasive carcinoma vs low-/ moderate-grade IPMNS
Cytokine panel	Maker et al. [148]	2011	40	HGD/invasive IPMN vs LGD/MGD	Il1β: 79%	Il1β: 95%	Il5, Il8, and Il1β levels were higher in advanced IPMN; Il1β remained accurate in differentiating the groups on multivariate analysis

Proteomics

There have been several small studies using proteomics-based approaches to detect differential protein expression in pancreatic cyst fluid to differentiate cyst types. In one study of 59 patients, a cluster of 14 proteins could differentiate serous cystadenomas from IPMN in 92%. Most tested proteins were downregulated in IPMN compared with serous cystadenoma [123]. In a small study of cyst fluid from 10 patients, 12 protein peaks were differentially expressed in pancreatic cyst fluid in patients with pancreatic adenocarcinoma compared to nonmalignant cysts [124]. In a study of surgical samples from IPMN in 43 patients and 952 protein peaks, 31 peaks were expressed differentially in IPMN with low to moderate dysplasia versus IPMN with severe dysplasia or invasive adenocarcinoma [125]. In the same study, results from five unspecified proteins were accurate in differentiating the groups with an AUC of 0.88 on receiver operator curve (ROC) analysis [125]. These data are promising and will hopefully identify targets appropriate for further study.

MUC Proteins

Mucin (MUC) glycoproteins are involved in lubricating and enforcing the epithelial lining of luminal organs, including the pancreatic duct [126]. Interest in MUCs as biomarkers arises from our understanding that MUC expression varies by IPMN histologic subtype, which affects malignant potential. Gastric-type IPMN, comprising the majority of branch-duct IPMN, expresses MUC5AC but not MUC1/2, and rarely exhibits high-grade dysplasia. The intestinal type of IPMN that makes up the majority of the main-duct IPMN expresses MUC2, often exhibits intermediate- to high-grade dysplasia, and is prone to developing invasive carcinoma. Pancreatobiliary-type IPMNs are rare, but typically express MUC1 and demonstrate high-grade dysplasia and often contain invasive or minimally invasive carcinoma [14, 126]. A panel of three glycan alterations on MUC5AC was shown to be able to differentiate mucinous from nonmucinous cysts with sensitivity of 87–89% and specificity of 100% [127]. Direct staining of cyst fluid for mucin expression yielded a sensitivity of 80% and a specificity of 40% for diagnosing mucinous cysts [128]. MUC2 and MUC4 concentrations were higher in cyst fluid in patients with IPMN with high-grade dysplasia or carcinoma compared to IPMN with low- to moderate-grade dysplasia [129]. Similarly, serum MUC5 AC was higher in patients with IPMN with high-grade dysplasia [129]. Overall, these data were collected from small samples of patients, retrospectively. However, in a more recent study with a training cohort followed by a prospective cohort of 68 patients, MUC5 AC and MUC2 expression from cyst fluid could discriminate premalignant and malignant cysts from benign cysts in 97% of cases [130]. In the same study, MUC5 AC and prostate stem-cell antigen could identify high-grade dysplasia and cancer with a 96% accuracy [130]. In another prospective study, proteomic MUC profiling was more accurate than cytology and cyst fluid CEA in identifying lesions with malignant potential and predicting malignant transformation [131].

In addition to MUC subtype expression in cyst fluid, analyses of the DNA methylation status of MUC1, MUC2, and MUC4 in pancreatic juice were useful in

differentiating PDAC from gastric- and intestinal-type IPMN in 45 patients [132]. Cytology from pancreatic duct lavage with staining for MUC1, MUC2, MUC5 AC, and MUC6 was useful in differentiating benign and malignant IPMNs with sensitivity and specificity of 92% and 100%, respectively [133]. Finally, MUC1 mRNA expression in pancreatic juice obtained from ERCP was higher in IPMN with carcinoma than in benign IPMN [134].

mAb Das-1

mAb Das-1 is a monoclonal antibody against a colonic epithelial phenotype that is reactive to premalignant conditions of the upper GI tract including Barrett's esophagus/esophageal adenocarcinoma, incomplete type gastric intestinal metaplasia/gastric adenocarcinoma, and small bowel adenomas/small bowel adenocarcinoma [135–138]. The specific antigen reactive to mAb Das-1 remains unknown, limited by its very high molecular weight (>200kd) and extensive glycosylation. Given the recent observations that intestinal-type IPMNs were at particular malignant potential and the utility of this biomarker in identifying colonic-type metaplasia in multiple organs, we sought to investigate the reactivity of this biomarker in patients with IPMN. In an initial study with 94 surgically resected IPMNs, mAb Das-1 was overexpressed in high-risk and malignant IPMN compared with low-risk IPMN (sensitivity 85%, specificity 95%) [139]. In the same study, mAb Das-1 expression was highly reactive in cyst fluid obtained perioperatively from high-risk/malignant IPMN, but minimally reactive in low- and intermediate-grade IPMN, yielding a sensitivity and specificity for detecting high-risk/malignant IPMN of 89% and 100%, respectively [139]. Further multicenter validation studies are currently underway.

Sonic Hedgehog

Sonic Hedgehog (SHH) is detected in IPMN cyst fluid, but not in pancreatic juice associated with chronic pancreatitis [46, 140]. SHH expression was more commonly expressed in surgical specimens with invasive carcinoma or high-grade IPMN than in moderate- or low-grade IPMN [141, 142]. In a systematic review by Tanaka et al., surgical pathology stained for SHH in 148 IPMNs yielded a sensitivity of 81% and specificity of 66% in differentiating HGD, CIS, and invasive carcinoma from benign IPMN [65].

S100

The S100 family is a group of proteins involved in cell signaling with increased expression in PDAC and IPMN associated with PDAC. One member was associated with nodal spread in PDAC [143]. In one study of mRNA expression in bulk tissue and pancreatic juice, S100P, a specific member of the S100 family, was useful for differentiating neoplastic disease from chronic pancreatitis; however, further studies are needed [144].

Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a product of the inflammatory pathways, and overexpression has been observed in multiple cancer types, including pancreatic cancer [145]. PGE_2 in cyst fluid was higher in IPMN than other mucinous cystic neoplasms and increased linearly by dysplastic grade from low-grade through invasive carcinoma [146]. In a larger prospective study of 100 patients with IPMN, PGE_2 levels in cyst fluid were higher in high-grade and invasive IPMN than low-/moderate-grade IPMN. In a subset of patients with cyst fluid CEA > 192 ng/ml, PGE_2 at a threshold of 0.5 pg/μl yielded a sensitivity of 78%, specificity of 100%, and accuracy of 86% for detecting high-grade or invasive IPMN [145].

Other Protein-Based Biomarkers

Plectin-1 (Plec-1) is highly specific and sensitive for early invasive PDAC [147]. In one small retrospective study, Plec-1 expression had a sensitivity of 84% and a specificity of 83% in differentiating malignant IPMN from benign IPMN, though further studies are required for validation [147].

In an analysis of cytokine expression profiles in IPMN cyst fluid, IL5, IL8, and IL1β concentrations were higher in cyst fluid from patients with HGD or cancer than in patients with low or moderate dysplasia [148]. At a level >1.26 pg/ml, the sensitivity was 79% and specificity was 95% [148]. On multivariate analysis, IL1β remained a significant predictor of high-risk cysts with an AUC of 0.92 [148].

Amphiregulin (AREG), an epidermal growth factor ligand overexpressed in pancreatic cancer, was significantly higher in cyst fluid in pancreatic cysts with cancer in a retrospective, single-center study [149]. At a threshold of 300 pg/ml, AREG had a diagnostic accuracy of 78% for cancer or high-grade dysplasia in cysts of multiple types with a sensitivity of 83% and a specificity of 73% [149].

Serine protease inhibitor Kazal type 1 (Spink1) is a peptide that has been associated with ovarian, bladder, and renal cancers. In a study of 61 surgical patients with various pancreatic cystic lesions, Spink1 levels were higher in surgically recommended lesions (main/mixed-duct IPMN and mucinous cystadenoma) than in benign lesions (side-branch IPMN and serous cystadenoma) [150]. At a cutoff of 118 μg/l, the sensitivity for differentiating surgically recommended lesions from benign lesions was 85% with a specificity of 84% and AUC of 0.94 [150].

Metabolomics

Metabolites in cyst fluid have been measured as a biomarker to differentiate mucinous from nonmucinous cysts. In an exploratory study of 45 cysts, concentrations of glucose and kynurenine, a tryptophan metabolite associated with cancer

development, were lower in mucinous cysts with high accuracy (AUC 0.92 and 0.94, respectively) [62, 151]. However, these tests could not distinguish premalignant from malignant cysts [151]. In another study, glucose was lower in mucinous cysts compared to nonmucinous cysts, though this was not examined as a marker of invasiveness [152].

Biomarkers in Other Tissues (Table 3.5)

Blood Parameters

An increased serum neutrophil to lymphocyte ratio (NLR) may be indicative of increased active inflammation in IPMN-derived malignancy as it is associated with tumorigenesis in other systems [48]. In two retrospective studies, the preoperative NLR was higher in patients with IPMN with carcinoma than with IPMN alone [153, 154]. In a recent study of 205 IPMNs, the sensitivity and specificity for NLR in detecting high-grade dysplasia and invasive carcinoma were 35.3% and 87% [155]. Even when combined with CEA and Ca19-9, the combination assay yielded a modest sensitivity of 58.8% and a specificity of 76.8% [155].

Pancreatic Juice

Analysis of pancreatic juice is attractive as it potentially represents a sampling of the entire network of pancreatic ducts. Given the inherent connection and genesis of IPMN from ductal cells of the pancreas, pancreatic juice analysis may allow us to more accurately assess the entire organ and overcome issues of "field defect" that have been well demonstrated in IPMN and IPMN-associated PDAC. Using sampling of pancreatic juice aspirated during ERCP preoperatively for NGS, TP53 was detected in 50% of malignant IPMN [156]. The concentration of SMAD4/TP53 mutations in secretin-stimulated pancreatic juice collected from the duodenum could distinguish PDAC from IPMN with 32.4% sensitivity and 100% specificity [157]. In this study, 50% of the patients who developed cancer despite close surveillance had SMAD4/TP53 mutations detected in pancreatic juice samples 1 year prior to cancer detection [157].

Stool

In a proof-of-concept study to detect gene mutations in stool for early detection of pancreatic cancer, BMP3 detected 51%, KRAS detected 50%, and the combination of mutations in either gene detected 67% of pancreatic cancers [158]. This has not been evaluated prospectively or in invasive IPMN.

Table 3.5 Non-cyst fluid biomarkers

Biomarker	Study	Year	Sample size	Definition of advanced neoplasia	Sensitivity	Specificity	Other
Neutrophil to lymphocyte ratio	Arima et al. [153]	2015	76 IPMN	IPMN with carcinoma vs IPMN with adenoma	NLR > 2.074: 73.1% Combined criteria: 27%	NLR > 2.074: 58% Combined criteria: 96%	NLR > 2.074: PPV 47.5% and NPV of 80.6% for combined criteria of ICCS 2012 guidelines NLR > 2.074 and Ca19-9 > 37 IU/ml: PPV 78% and NPV of 72%
Neutrophil to lymphocyte ratio	Gemenetzis et al. [154]	2016	272	IPMN with carcinoma	NLR > 4: 33.8% NLR > 2.65: 74.1%	NLR > 4: 95% NLR > 2.65: 83.1%	NLR > 4: PPV 70.6% and NPV 80.3% Model including NLR, imaging findings, and jaundice: sensitivity 43.8% to 96.5% and specificity 98% to 50.3%
NLR, CRP to albumin ratio (CAR), CEA, CA19-9	Hata et al. 2019 [155]	2019	205	HGD /invasive carcinoma	NLR > 2.5: 35.3% CAR > 0.03: 31.8% CEA > 5, Ca19-9 > 37, and NLR: 58.8%	NLR > 2.5: 87% CAR > 0.03: 82.6% CEA > 5, Ca19-9 > 37, and NLR: 58.8%	NLR > 2.5: PPV 84.2% CAR > 0.03: PPV 77.8%, CEA > 5, Ca19-9 > 37, and NLR: PPV 83.3%
Pancreatic juice NGS	Yu et al. [157]	2017	115	PDAC vs IPMN	32.4%	100%	NGS of mutant TP53/SMAD4

Stool DNA	Kisiel et al. [158]	2012	57 cases, 62 controls	Pancreatic cancer	BMP3: 51% KRAS: 75% Combination: 67%	BMP3: 90% KRAS: 90% Combination: 90%	
Circulating epithelial cells	Rhim et al. [160]	2014	48	PDAC vs pancreatic cysts vs controls			>3 CECS in 73% with PDAC, 33% with pancreatic cysts and 0 controls
Circulating epithelial cells	Poruk et al. [161]	2017	19 with IPMN without malignancy	IPMN vs other cysts	58%	100%	IPMN with HGD more likely to have dual staining CECs

Circulating Cells

Preclinical mouse models of pancreatic cancer have demonstrated that epithelial to mesenchymal transition is indeed an early event in the development of PDAC, and circulating pancreatic tumor cells may in fact be present in the bloodstream prior to the demonstration of overt metastasis [159]. In a prospective study, 78% of patients with PDAC, 33% of patients with pancreatic cysts, and 0% of the controls undergoing screening colonoscopy had circulating epithelial cells (CECs) [160]. The outcome of those patients or their ultimate pathology was not reported in this study, nor was a threshold of "acceptable" circulating cells. In another study of patients undergoing surgical resection for IPMN, the sensitivity and specificity of cytokeratin-positive CECs for differentiating IPMN from other cysts were 58% and 100%, respectively [161]. In this study, CECs were more likely to be found in patients with high-grade dysplasia [161]. More recently, a group improved the assay and detected CECs in 88% of patients with IPMN regardless of underlying grade [162].

Another potential blood-based biomarker paradigm is analysis of circulating DNA for mutations seen in pancreatic tissues. In a retrospective study, the total amount of cell-free DNA was higher in patients with pancreatic cancer and IPMN than in controls [163]. GNAS mutations were detected in the cell-free DNA from patients with IPMN, but not serous cystadenomas or controls [163]. In a proof-of-concept study, KRAS and TP53 mutations in serum were detected in PDAC and IPMN, and KRAS mutations were detected in chronic pancreatitis patients [164]. While very exciting, these studies remain investigational and will hopefully be able to be integrated with other genetic/protein biomarkers to develop a minimally invasive surveillance tool for patients with pancreatic cysts or who are otherwise at risk for pancreatic cancer.

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Part III
Diagnosis, Biomarkers and Stratification

Chapter 4
Challenges and Opportunities for Early Pancreatic Cancer Detection: Role for Protein Biomarkers

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The Case for Earlier Detection

Currently, for three out of four PDAC patients, the diagnosis of PDAC comes at a time when the disease is advanced. Late diagnosis severely limits treatment options and contributes to the poor overall 5-year survival of 7%. Reliable diagnostic biomarkers that facilitate earlier diagnosis are much needed [1]. Generally speaking, it is recognised that early detection of cancer increases the opportunities for effective management and treatment (http://www.who.int/cancer/prevention/diagnosis-screening/en). Biomarker development projects aim to introduce diagnostic biomarkers that will permit PDAC detection at a time when therapeutic intervention that leads to improved prognosis is feasible. It is essential to ensure that earlier detection facilitates interventions that both improve outcome and well-being for patients, without simply increasing the time interval between diagnosis and death, known as lead time. Much research supports the benefit of earlier PDAC detection. Patients in whom PDAC is incidentally diagnosed have longer median survival compared with PDACs discovered when patients are symptomatic [2]. Furthermore, patients diagnosed with stage I disease survive markedly better compared to patients with all other stages [2]. Surgery followed by chemotherapy confers a significant survival advantage [3, 4]. In this setting, the 5-year survival is 70% for stage I disease and 22% for stage III disease. The ESPAC-4 trial which compared

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gemcitabine to combination gemcitabine/capecitabine therapy in R0/R1 resected PDAC patients demonstrated superior 5-year survival of 28·8% in the gemcitabine/capecitabine arm [5]. By contrast, for patients with locally advanced and metastatic pancreatic cancer randomised to either gemcitabine or gemcitabine/capecitabine, the 1-year survival in the superior arm of gemcitabine/capecitabine was 24% [6]. Thus, detecting PDAC at a time when patients are eligible for potentially curative surgery or for neoadjuvant therapy to downstage locally unresectable disease could significantly improve prognosis. The success of early detection schemes will require education of both the general public and healthcare professionals so that possible warning signs of pancreatic cancer are recognised. Alongside education, effective screening will be a major element in earlier detection of PDAC.

Challenges Associated with Diagnostic PDAC Marker Development

PDAC early detection faces a number of critical challenges (Table 4.1). PDAC is relatively uncommon, and it is therefore difficult for a single group or institution to amass the number and variety of samples required for successful biomarker development. PDAC tumours exhibit both intra-tumour and inter-individual variation [7,

Table 4.1 Challenges and potential solutions to early detection biomarker development for pancreatic cancer

Description	Challenge	Potential solutions
PDAC is a relatively uncommon disease	Large numbers of samples are required for novel biomarker development	National and international collaboration is required for adequate sample availability
PDAC tumours exhibit both intra-tumour and inter-individual variation	Large numbers of samples are required to enable diversity to be captured	National and international collaboration is required Capture as much clinico-pathological data relevant to samples as possible Sub-categorise samples to allow biomarker performance in individual categories to be manifested
PDAC is accompanied by comorbidities	Comorbidities such as obstructive jaundice, new-onset diabetes mellitus and chronic pancreatitis could influence biomarker behaviour or mislead biomarker analysis (e.g. the biomarker detects the comorbidity rather than the principal disease)	Carefully design studies Include groups that control for comorbidities, allowing the biomarkers' power to discriminate cancer from controls to be accurately assessed Be clear about the intended use population

Table 4.1 (continued)

Description	Challenge	Potential solutions
The majority of PDAC patients are diagnosed with late-stage disease	Use of late-stage samples in biomarker discovery and/or validation may lead to the detection of biomarkers capable of detecting late-stage disease, but not necessarily early-stage disease	Include patients with early-stage disease and studies Use pre-diagnostic samples where possible Collect custom-made bespoke cohorts in order to obtain pre-diagnostic samples with pancreatic cancer detection specifically in mind

8]. This heterogeneity is likely to be reflected in a variation in biomarkers from patient to patient, and robust biomarker panels may be required. PDAC is often accompanied by obstructive jaundice, which can lead to false-positive findings in blood-borne biomarker studies [9–11]. Moreover, PDAC-associated diabetes is present in a substantial proportion of individuals with pancreatic cancer [12]. Therefore, it is conceivable that biomarkers appearing to relate to PDAC could be the consequence of diabetes and as such may be present in cancer-free individuals who have diabetes. Finally, PDAC tissue exhibits areas of chronic pancreatitis. Understanding the impact of comorbidities on PDAC biomarkers is essential and requires carefully designed studies. Depending on the intended use population, samples from multiple disease controls may be required.

To date, most studies aimed at identifying early-stage biomarkers of PDAC have used samples from patients already diagnosed with PDAC and are thus compromised by both late changes during tumorigenesis that are not seen in early-stage disease and the general poor health of patients with advanced disease. It is recognised that new lines of early detection research should include relevant early-stage, pre-diagnostic samples in order to validate existing biomarkers and offer chances of discovering new biomarkers of early PDAC. Some of the protein markers discussed below have been discovered or validated in such samples.

How Would a Biomarker Panel Be Used?

The intended use of a biomarker will dictate the required sensitivity and specificity and the patient and control groups required during biomarker development. Individual and tumoural heterogeneity suggests that no single biomarker on its own will give adequate sensitivity and that a panel of two or more protein biomarkers will be required. Moreover, if the biomarker is being used to stratify risk within a population, then it is likely that follow-up screening will be carried out to achieve diagnosis (Fig. 4.1). Thus, while high sensitivities and specificities are desirable, currently it is envisaged that a positive biomarker test will not be used as a stand-alone diagnostic.

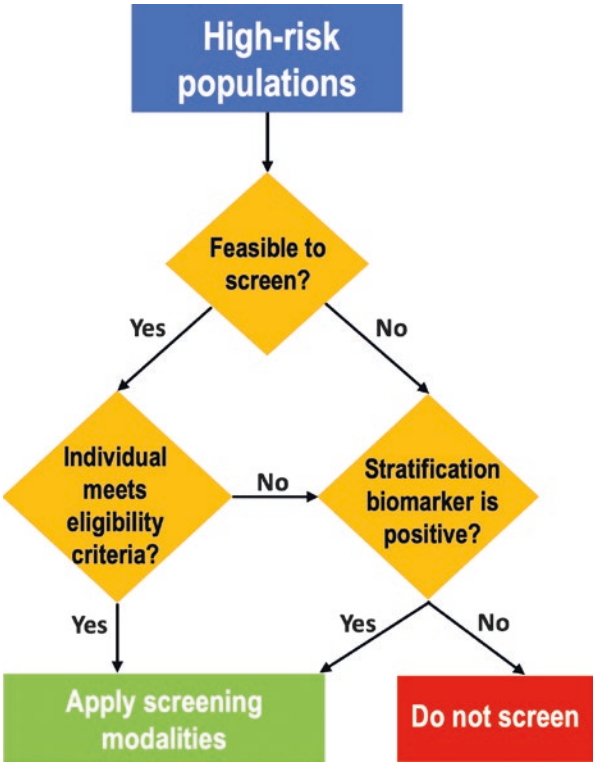

Fig. 4.1 Pathways to screening in high-risk groups; role of biomarkers. Currently there are no biomarkers suitable for screening the general population. Although PDAC is a leading cause of cancer deaths, it is relatively uncommon. A screening test would have to be extremely specific (approaching 100%) in order to avoid large numbers of false positives, and no such screening modality presently exists. For some high-risk populations, it is feasible to screen individuals who meet the eligibility criteria. However, even if individuals in this group do not meet the eligibility criteria, a positive biomarker result may suggest that screening via existing modalities (EUS, CT/MRI scan, biochemistry panels) is warranted. Within some high-risk groups, such as individuals with new-onset diabetes mellitus, subjecting all individuals to screening is not feasible due to the low incidence of PDAC within the population. Biomarkers for early detection of PDAC could select for those at a higher risk of a PDAC diagnosis, creating an enriched group of the highest-risk individuals to be screened. Those with a negative result from the biomarker test and therefore deemed to be at lower risk would be spared the worry and inconvenience of screening, while the healthcare system would avoid the associated burden and costs of unnecessary screening

Barriers to Screening for PDAC

Until relatively recently, cancers, such as breast and cervical cancer, seemed intractable. However, mortality from these cancers has decreased, in part attributed to the introduction of screening programmes which facilitate detection of early lesions or localised tumours that are easier to treat. There are practical barriers to screening the general public for PDAC. Although the mortality rate of PDAC is very high, the

disease is relatively uncommon, with an incidence in Europe of 8/100,000 (Age Standardised Rate) [13]. Given the diagnostic accuracy of current detection methods, this is too low to permit screening of the asymptomatic adult population. False positives for this disease are especially serious, as it is not easy to access pancreatic lesions. For some patients, a definitive diagnosis requires surgery, and this carries the risk of significant morbidity and mortality. There are at least two ways in which the accuracy of screening for PDAC could be improved. Firstly, the development of a high-performing screening test could make screening possible. Secondly, restricting screening to those at the highest risk of PDAC would increase the rate of disease detection and reduce the occurrence of false-positive findings. These two options are not mutually exclusive, and implementing a higher-performing screening test in a high-risk population would offer the best chances of increasing accuracy [2]. Effective screening requires many conditions to be met, including that its effectiveness is proven, that it is resourced sufficiently to cover the group being screened, that a pathway exists for confirming diagnoses and for offering treatment and follow-up where tests show abnormal results, and, finally, that the prevalence of the disease should be sufficiently high to justify the costs of screening. The cost-benefit analysis should take into account the cost of the initial screening test as well as the cost of subsequent tests required to confirm the diagnosis. Since, in all cases, abnormal results require confirmatory tests, keeping false positives to a minimum is essential to reduce costs. Thus, great attention needs to be paid to the specificity of the test. Ghatnekar et al. [14] describe a model which enabled them to determine cost and the quality-adjusted life years (QALY) of screening for PDAC using a biomarker panel. According to their model, screening high-risk individuals for PDAC using a serum biomarker panel is highly desirable.

High-Risk Groups

An estimated 10% of patients with PDAC have a family history of the disease. For a proportion of these families, the pattern of risk is consistent with autosomal dominant predisposition [15]. In Europe, the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) is the largest registry of families with an inherited risk of PDAC. The United States also has successful pancreas screening [16]. Currently, screening is restricted to families with an inherited risk [17]. By contrast, 90% of PDAC cases cannot be predicted by family history and are considered sporadic. No current screening modality exists for sporadic PDAC.

Epidemiological data indicate that PDAC can cause diabetes mellitus [18], with new-onset diabetes an early warning sign of the presence of PDAC [18]. Sharma et al. reported that pancreatic cancer patients are hyperglycaemic for an average duration of 36–30 months before PDAC diagnosis [19]. At the time of PDAC diagnosis, the majority of PDAC patients have diabetes [12, 20]. By contrast, the prevalence of diabetes in individuals with lung, breast, prostate, and colorectal cancers is no higher than non-cancer controls [21]. New-onset diabetes

occurs in ~50% of PDAC cases; it is the largest high-risk group for sporadic PDAC. With regard to early detection, distinguishing new-onset diabetes caused by PDAC (known as type 3c) from the more common type 2 form of the disease would allow for earlier diagnosis of PDAC [18]. However, hyperglycaemia and diabetes are common in the general population, and additional factors will need to be considered in order to enrich individuals within this group who are most likely at risk of being diagnosed with PDAC. Based on data from four independent cohorts of patients with new-onset diabetes, Sharma et al. identified three factors which were strongly correlated with PDAC [22], change in weight, change in blood glucose, and age at onset of diabetes. These form the basis of the Enriching New-Onset Diabetes for Pancreatic Cancer (ENDPAC) model. Biomarkers may allow for further enrichment of the new-onset diabetes group for PDAC.

The Need for Better Biomarkers

CA19–9 is the only biomarker in routine use for the management of PDAC [23, 24]. It has a number of limitations including lack of expression in ~5% of the population and elevation in related diseases including chronic pancreatitis and obstructive jaundice [23, 25]. CA19–9 has a sensitivity/specificity of ~85%/~85% for the detection of advanced PDAC [26]. Since PDAC is relatively uncommon, screening the general population with CA19–9 is not feasible because for every true positive identified, several thousand false positives would also be identified. All positives (both true and false) would require additional tests (imaging, biochemical panels) to verify the presence of PDAC. The ratio of true positives to false positives is far too low to justify the costs of additional tests and the potential harm caused to individuals without the disease who test positive for it. Consequently, biomarkers with superior sensitivities and particularly superior specificities are required.

Progress in Protein Biomarker Development

A number of recent studies which reported protein biomarkers are compiled here (Table 4.2). Given the large body of literature to select from, we have prioritised studies which have either used pre-diagnostic samples, included samples from early-stage disease cases, or contained large numbers of subjects. Additionally, we have made subjective decisions about the studies that are most relevant to our own research interests. Liquid biopsy denotes a sample of body fluid collected in a minimally invasive manner [27]. The liquid biopsy most frequently analysed for biomarkers of PDAC is blood (Table 4.2), although other body fluids such as urine and saliva have also been investigated.

Table 4.2 Selected blood-borne protein biomarkers

Protein biomarkers	Analysis included	Performance	Sample source	Reference
CA19–9 and TSP-1	Multiple reaction monitoring (MRM)	AUC of 0.86 to distinguish PDAC (in samples taken between 0 and 24 months prior to diagnosis) from control	Blood; pre-diagnostic PDAC cases (UKCTOCS), chronic pancreatitis, healthy controls, diagnosed PDAC, KPC mice	Jenkinson et al. [28]
ERBB2, ESR1 and TNC	Antibody microarray	AUC of 0.86 for diagnosed PDAC; AUC of 0.68 for the pre-diagnostic samples	Blood; KPC mice, pre-diagnostic plasma samples from women in the Women's health initiative (WHI)	Mirus et al. [29]
CA19–9	ELISA and/or CLIA	At 95% specificity, the sensitivity of CA19–9 (>37 U/mL) was 68% up to 1 year, and 53% up to 2 years prior to diagnosis	Blood; pre-diagnostic PDAC cases (UKCTOCS)	O'Brien et al. [30]
LYVE-1, REG1A, and TFF1	GeLC/MS/MS, ELISA	AUC of 0.92 to distinguish stage I and II PDAC cases from healthy controls	Urine; PDAC samples, including with early stage, healthy control, chronic pancreatitis	Radon et al. [31]
CA19–9 with THBS2	ELISA	AUC >0.84 to distinguish PDAC of all stages from controls	Blood; PDAC samples, including with early stage, healthy control, chronic pancreatitis	Kim et al. [32]
29-protein biomarker panel	Antibody microarray	AUC of 0.96 to distinguish stage I and II PDAC cases from healthy controls	Blood; PDAC samples, including with early stage, healthy control, chronic pancreatitis	Mellby et al. 2018 [33]

AUC area under the curve, *GeLC-MS/MS* SDS-PAGE-Liquid Chromatography-Tandem Mass Spectrometry, *UKCTOCS* United Kingdom Trial of Ovarian Cancer Screening

Future Perspectives

There is no doubt that improvements have been made in the way in which biomarkers are discovered and validated. A key issue for biomarker programs that use samples from individuals already diagnosed with PDAC is gauging whether biomarker alterations, evident at the time of diagnosis, are detectable earlier in the disease pathway. A number of cohort studies containing samples taken from individuals who went on to be diagnosed with PDAC show that certain candidate markers perform poorly in pre-diagnostic samples. Lokshin and co-workers [34] used pre-diagnostic sera from PDAC patients from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) to evaluate the performance of 67 proteins. They concluded that most biomarkers identified in previously conducted case/control studies are ineffective in pre-diagnostic samples, including examples such as MIC-1, TIMP-1, ICAM1, HE4, OPG, MUC1, and MMP9. Jenkinson et al. [35]

similarly reported that ICAM-1 and TIMP-1, promising candidate PDAC diagnostic markers, failed to show significant elevation in samples from the UKCTOCS study taken 0–12 months prior to PDAC diagnosis [35]. Both ICAM-1 and TIMP-1 proteins were significantly elevated in blood from PDAC patients with obstructive jaundice [35], and this finding possibly explains the observed upregulation of these proteins in diagnosed PDAC cases.

The use of existing cohorts for the discovery or validation of PDAC early detection biomarkers is not ideal. Existing cohorts lack important demographic data such as diabetes status, presence of obstructive jaundice, or history of chronic pancreatitis. For this reason bespoke pre-diagnostic cohorts are currently being assembled. In the United States, Chari and colleagues in the Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer (CPDPC) have begun to assemble a prospective high-risk cohort of 10,000 individuals with new-onset diabetes mellitus, called NOD [36]. In the United Kingdom, a similar cohort of 2500 individuals, called UK-NOD, is being led at University of Liverpool by the authors of this book chapter, and there are other similar initiatives underway in Europe. Together, these multi-centre collaborative projects have the scale to acquire the high numbers of individuals (with presymptomatic PDAC and new-onset type 2 diabetes mellitus) necessary for rigorous validation of existing biomarkers and the discovery of new early detection biomarkers for PDAC. High-risk registries of familial PDAC will also provide an invaluable resource for the development of early PDAC biomarkers. It is foreseeable that biomarkers for early detection of PDAC will initially be tested and used in high-risk groups. This progress in early detection, along with concurrent advances in treatment, will undoubtedly lead to improvements in outcomes for PDAC patients.

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Chapter 5
Metabolic Biomarkers of Pancreatic Cancer

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The Metabolome: Mirror of Our Organism

Since the completion of sequencing of the human genome, several systemic profiling tools have been developed to provide a more comprehensive picture of tumor development [1]. The acquisition of cancer hallmarks necessitates molecular alterations at multiple levels including genome, epigenome, transcriptome, proteome, and metabolome [2]. The different "-omics" levels vary greatly in their complexity which is largely driven by spatial and temporal dynamics, chemical modifications, and environmental influence. The flow of information from genome to protein and ultimately to metabolites is accompanied by an exponential increase in the complexity [2, 3]. Though functional genomic strategies such as transcriptome and proteome led to the understanding of cancer biology, such as the identification of new tumor subtypes and transcriptional and protein biomarkers for certain types of cancer [4–8], metabolic profiling provides the closest link to the phenotype of an organism. It has been known for almost a century that altered cell metabolism is a characteristic feature of cancers [1, 9, 10]. Aside from well-described changes in nutrient consumption and waste excretion, altered cancer cell metabolism also leads to changes in intracellular metabolite concentrations. Increased levels of metabolites that result directly from genetic mutations and cancer-associated modifications in protein expression can promote cancer initiation and progression [11].

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Techniques that monitor and discover metabolic changes in subjects related to disease status or in response to a medical or external intervention have great potential to impact clinical practice [12–14]. Measuring metabolite concentrations is a more sensitive approach than following the rates of chemical reactions directly. Metabolic control analysis has demonstrated that although changes in enzyme concentrations and activities have a small impact on metabolic flux, changes in flux have a significant impact on metabolite concentrations [15, 16]. This is because the control of metabolic flux of a pathway is spread across all enzymes present in the pathway, rather than being controlled by a rate-determining step. Furthermore, there is not necessarily a good quantitative relation between transcription and enzymatic activities. As metabolites are downstream of both transcription and protein synthesis cascade, they are potentially a better indicator of enzyme activity [1, 17]. Thus, metabolic profiling offers a particularly sensitive method to monitor changes in a biological system, through observed changes in the metabolic network.

Metabolic profiling is usually referred to as the quantitative study of a group of metabolites that are associated with a particular pathway [13]. Global metabolic profiling has been referred to as metabolomics. Metabolomics is defined as a quantitative description of all endogenous low-molecular-weight components (<1 kDa) in a biological sample using state-of-the-art analytical instrumentation in conjunction with pattern recognition techniques. Each cell type and biological fluid has a characteristic set of metabolites that reflect the organism under a particular set of environmental conditions and that fluctuate according to physiological demands [12]. Lipidomics is a specialized subset of metabolomics that evaluates lipid profiles [18]. Lipids play many important roles in cancer processes including invasion, migration, and proliferation [19].

Currently, a truly global comprehensive assessment of the metabolome by a single analytical platform is not yet possible, due to the high heterogeneity of the metabolites (chemical structure, physicochemical properties, and concentration) [20]. Although it would be ideal to know the entire metabolic content of a sample, depending on the purpose of analysis, there might be situations where the information provided from only one part would be sufficient; hence, the quantification and identification of “all” metabolites would not be necessary [21, 22].

Application of Metabolomics in Pancreatic Cancer Biomarker Discovery

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies and burdened with a 5-year survival rate of only 8% [5, 23]. Multiple factors are known to contribute to this dismal prognosis, most prominently delayed diagnosis and resistance to chemo- or radiation therapy [24]. Surgical resection alone, which is feasible in around 20% of patients, results in 5-year survival of around 10% [25]. The use of adjuvant chemotherapy with either 5-fluorouracil-folinic acid (5FU/folinic acid) or gemcitabine increases 5-year survival to around 28%, and the use of

FOLFIRINOX prolongs median survival to 54 months [26]. Even though insights into the molecular pathology of cancer can create opportunities for the development of therapies with substantial clinical benefit [27], for pancreatic cancer such options are currently unavailable [26, 28]. Biomarker-driven treatment strategies are urgently needed for PDAC [27]. Earlier diagnosis is one factor that could alter this trajectory [29].

The ultimate goal of most metabolomics cancer studies is to discover cancer-specific diagnostic, prognostic, or predictive biomarkers for a patient [30]. The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to exposure or intervention, including therapeutic interventions" [31]. Diagnostic biomarkers are used for the critical determination of whether a patient has a particular medical condition for which treatment may be indicated or whether an individual should be enrolled in a clinical trial studying a particular disease. A prognostic biomarker is one that indicates an increased (or decreased) likelihood of a future clinical event, disease recurrence, or progression in an identified population, while a predictive biomarker is used to identify individuals who are more likely to respond to exposure to a particular medical product or environmental agent. The response could be a symptomatic benefit, improved survival, or an adverse effect [32]. An ideal biomarker should meet various criteria that include the following: (i) it should be present in readily available and minimally invasive sources (e.g., blood and urine); (ii) it should be highly sensitive (allowing early diagnosis) and specific (unaffected by external and comorbid conditions); (iii) it should vary promptly in response to treatment and disease progression; (iv) it should provide a deeper understanding about the disease mechanism; and (v) it should be useful in risk stratification and prognosis [33]. Metabolomics has an advantage over other "-omics" and is better suited for this purpose. In fact, as changes in metabolites normally appear in readily available biofluids, such as blood and urine, the translation of metabolomic studies to clinical practice is easier [34]. Biofluids are usually the easiest samples to work with, requiring less sample preparation than other biological samples [21].

The metabolome is highly dynamic, reflecting continuous fluxes of both metabolic and signaling pathways, and is sensitive to diverse host and environmental factors. These unique features make metabolomics able to capture a plurality of subtle changes. Thus, metabolomics holds the promise for simultaneously evaluating a variety of complex pathways and their consequences [34]. Also, metabolomic experiments are also less expensive than proteomic and transcriptional approaches [21, 30, 35, 36].

Although targeting cancer metabolism is a promising therapeutic strategy, clinical success will depend on accurate diagnostic identification of tumor subtypes with specific metabolic requirements. Through broad metabolite profiling, PDAC was successfully categorized into three highly distinct metabolic subtypes, namely, slow proliferating, glycolytic, and lipogenic subtypes [37]. One subtype was defined by reduced proliferative capacity, whereas glycolytic and lipogenic subtypes showed distinct metabolite levels associated with glycolysis, lipogenesis, and redox

pathways. The lipogenic subtype associated with the epithelial subtype, whereas the glycolytic subtype strongly associated with the mesenchymal subtype, suggesting functional relevance in disease progression [37, 38]. This identification of distinct metabolic subtypes in PDAC may add to patient selection for investigational metabolic inhibitors and in the selection of new therapeutic targets [37, 39, 40].

Pancreatic Cancer-Specific Metabolic Biomarkers

The only routinely used serum marker for PDAC with demonstrated clinical usefulness for therapeutic monitoring and early detection of recurrent disease after treatment in patients with known PDAC is carbohydrate antigen 19–9 (CA19–9) [41]. Elevation of CA19–9 indicates advanced PDAC and poor prognosis [42, 43]. However, elevation of CA19–9 is observed in only 65% of patients with resectable PDAC [42, 44] and can also be caused by other conditions such as pancreatitis, cirrhosis, and cholestasis [45]. In addition, patients who are negative for Lewis antigen a or b (approximately 10% of patients with PDAC) are unable to synthesize CA19–9 and express undetectable levels, even in advanced stages of the disease. Although measurement of serum CA19–9 levels is useful in patients with known pancreatic cancer, the use of this biomarker as a screening tool has had disappointing results and is not recommended [41, 42, 46].

There are several attempts for metabolomics-based clinical investigations to identify potential biomarkers for diagnosis, stratification of a prognosis, and monitoring of therapy [47]. Table 5.1 summarizes studies that specifically addressed metabolic biomarkers for PDAC.

Table 5.1 Different metabolic biomarkers

Metabolites	Sample matrix	Discrimination group	Sample size	Conclusion	References
Plasma free amino acids					
Arginine ↓, total amino acids ↓	Plasma	PDAC vs control	21/21	Arginine decreased in cancer patients both with and without weight loss, irrespective of tumor type and stage	[48, 49]
PFAA index (serine ↓, asparagine ↑, isoleucine ↓, alanine ↓, histidine ↓, and tryptophan ↓)	Plasma	PDAC vs control	120/600	AUC on ROC analysis of PFAA index to discriminate PDAC from control was 0.89 (95% CI: 0.86–0.93)	[50]

Table 5.1 (continued)

Metabolites	Sample matrix	Discrimination group	Sample size	Conclusion	References
PFAA index (serine ↓, asparagine ↑, isoleucine ↓, alanine ↓, histidine ↓, and tryptophan ↓)	Plasma	PDAC vs CP vs control	240/28/7772	AUC on ROC analysis of PFAA index to discriminate PDAC from control were 0.81 (95% CI: 0.75–0.86) and 0.87 (95%CI: 0.80–0.93) to discriminate PDAC from CP	[50]
Total amino acids ↓	Plasma	PDAC vs CP vs control	12/12/12.	A significant deficit in circulating amino acid levels in pancreatic cancer patients	[51]
Xylitol ↓, 1,5-anhydro-D-glucitol ↓, histidine ↓, and inositol ↑	Serum	PDAC vs control	43/42	High sensitivity (86.0%) and specificity (88.1%) for PDAC	[52]
Xylitol ↓, 1,5-anhydro-D-glucitol ↓, histidine ↓, and inositol ↑	Serum	PDAC vs CP vs control	42/23/41	Displayed higher sensitivity (77.8%) in PDAC and lower false discovery rate (17.4%) in CP	[52]
Branched-chain amino acids					
Branched-chain amino acids ↑	Plasma	PDAC vs control	170/340	Increased branched-chain amino acids levels over a period of 10 years associated with increased incidence of PDAC	[55]
Branched-chain amino acids ↑, isoleucine ↑, leucine ↑, and valine ↑	Plasma	PDAC vs control	453/898	Elevated risk was independent of known predisposing factors, with the strongest association observed among subjects with samples collected 2 to 5 years before diagnosis	[54]

(continued)

Table 5.1 (continued)

Metabolites	Sample matrix	Discrimination group	Sample size	Conclusion	References
Choline-containing metabolites					
Glutamate ↓, choline ↓, betaine ↓, methyl-guanidine ↑, and 1,5-anhydro-D-glucitol ↓	Plasma	PDAC vs control	200/200	High sensitivity (97.7%) and specificity (83.1%) (AUC = 0.943, 95%CI = 0.908–0.977). Independent cohort showed satisfactory accuracy (AUC = 0.835; 95%CI = 0.777–0.893)	[60]
Glycolysis-related metabolites					
3-Hydroxybutyrate ↓, 3-hydroxyisovalerate ↓, lactate ↓, and trimethylamine-N-oxide ↓	Serum	PDAC vs control	17/23	Significant higher level of isoleucine, triglyceride, leucine, and creatinine in PDAC	[65]
3-Hydroxybutyrate ↓, 3-hydroxyisovalerate ↓, and lactate ↓	Plasma	PDAC vs CP vs control	19/20/20	Sensitivity of discrimination between PDAC and chronic pancreatitis is 84% with specificity of 90%	[66]

Amino Acids

Cancer cells require certain amino acids for DNA synthesis, building new blood vessels, and duplicating their entire protein content. These proteins work as growth-promoting hormones or tumor growth factors. The increase in the amino acid demand may thus lead to lower availability of plasma free amino acids as detected in cancer patients. Another possibility to explain decreased levels of amino acids is cancer-associated malnutrition. Patients with pancreatic cancer are usually troubled by malnutrition due to exocrine pancreatic insufficiency just to name on one possible explanation [48].

It has been reported that plasma free amino acid (PFAA) concentrations in PDAC, significant decreases arginine levels, regardless of tumor types and stages, weight loss or body mass index are specific features of the presence of a malignant tumor. Concomitantly, a decrease in total amino acids was detected [49]. Fukutake et al. [50] delineated a PFAA index comprising of serine, asparagine, isoleucine, alanine, histidine, and tryptophan as variables to calculate the PDAC risk and

successfully discriminate patients with PDAC from control subjects. Several other studies with small sample size [51, 52] reported similar decreases in circulating free amino acid levels in PDAC patients.

Branched-Chain Amino Acids

An accumulating body of evidence demonstrates that branched-chain amino acids (BCAAs), valine, leucine, and isoleucine are essential nutrients for cancer growth and are utilized by tumors in various biosynthetic pathways as a source of energy [9, 53]. It is known that elevated plasma levels of BCAAs are associated with a greater than twofold increased risk of future PDAC diagnosis. This elevated risk was independent of known predisposing factors, with the strongest association observed among subjects with samples collected 2–5 years before diagnosis, when occult disease is probably present [54]. In line, in the Japan Public Health Center-based prospective study, an association between increased plasma BCAA level and increased risk of pancreatic cancer, particularly when an increase in BCAAs was observed at least 10 years before diagnosis, was confirmed [55].

Choline-Containing Metabolites

Aberrant choline metabolism, characterized by increased phosphocholine and total choline-containing metabolites, is a primary cause of choline-containing metabolites due to an overexpression of the choline kinase-α (*CHKA*) and increased expression of choline transporters [56, 57]. PDAC cell lines and pancreatic tumors showed elevated choline-containing metabolites. Total choline-containing metabolites were observed as a single peak in vivo which in turn was resolved in proton spectroscopy to belong to the metabolites phosphocholine, glycerol-phosphocholine, and free choline [57]. However, in an animal study involving rats, choline-containing metabolites were decreased in PDAC [58]. Choline deficiency can also induce severe acute pancreatitis in animal models [59]. Reduced plasma betaine levels enhanced the discrimination of PDAC from control. Because choline is a precursor of betaine, the depletion of both betaine and choline in PDAC may be interrelated [47, 60]. A diagnostic model based on logistic regression incorporating a panel of five metabolites which constitutes choline and betaine (glutamate, choline, betaine, methylguanidine, and 1,5-anhydro-D-glucitol) robustly distinguished PDAC from normal controls [60]. FI-FTICR-MS metabolomic analysis showed significant reductions in serum levels of metabolites belonging to 36-carbon ultra-long-chain fatty acids; multiple choline-related systems including phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins; as well as vinyl ether-containing ethanolamines in PDAC patients if compared to controls [61].

Glycolysis Metabolites

Constitutively active components of the Ras pathway stimulate cellular glucose uptake and metabolic rate, hereby overcoming the capacity of the cell to utilize mainly glucose for its bioenergetic requirements. As a result, tumorigenic cells secrete excess metabolites of the glycolytic pathway in the form of lactic acid. Recent studies have strongly implicated aerobic glycolysis in the malignant etiology of pancreatic tumor cells [62, 63]. The shift to enhanced glucose metabolism in hypoxic pancreatic cancer cells is clearly manifested by the substantial accumulation of the glycolysis end-product lactic acid in the tumor microenvironment. Interestingly, PDAC patients frequently also suffer from diabetes and hyperglycemia, conditions typified by high blood sugar [63, 64]. Serum lactate levels tend to be higher in patients with malignancies [58]. In contrast, serum metabolic analysis revealed significantly lower 3-hydroxybutyrate, 3-hydroxyisovalerate, lactate, and trimethylamine-N-oxide in PDAC compared to that of controls and chronic pancreatitis [65, 66].

Lipid Metabolites

Saturated fatty acids are known to affect insulin secretion and insulin resistance, which might be involved in the carcinogenesis of the pancreas [67]. It has been shown that fatty acids may regulate cancer cells by modulating hypoxia-inducible factor-1 (HIF-1) which encodes for proteins including glucose transporters and growth factors. Several clinical studies demonstrated a significant association between saturated fat intake and pancreatic cancer [68]. Besides, Matters et al. have shown that dietary fat can induce growth and metastasis of pancreatic cancer [69]. Although risk factors in their complexity causing pancreatic cancer are still not fully understood, it is clear that high-fat diet is one of the risk factors associated with PDAC [70].

The two best-studied families of polyunsaturated fatty acids (PUFAs), linoleic acid (n-6) and α-linolenic acid (n-3), are essential fatty acids, and they exert opposite effects on cancer development. n-3 PUFAs can suppress tumor carcinogenesis by giving rise to pro-inflammatory eicosanoids, whereas n-6 PUFAs promote cancer development by giving rise to anti-inflammatory eicosanoids. It has been reported that higher consumption of n-3 PUFAs may protect patients against cancers [71]. Self-reported measures of dietary n-3 PUFAs intake generally derived from food frequency questionnaires (FFQ) are used to assess the n-3 PUFAs intake, but for conformation concentration of serum phospholipid, n-3 LC-PUFAs should be used [72]. Even though growing data, including from epidemiology studies, suggest that n-3 LC-PUFAs may have a protective role on PDAC, the role of n-3 LC-PUFAs as biomarkers and the relationship between these biomarkers and the disease still need to be explored [73]. Lipidomics revealed significant alteration in PDAC for four

significant metabolite families, 36-carbon long-chain fatty acid, lysophosphatidylcholine, phosphatidylcholine, and sphingomyelins [74]. PC-594 is a novel circulating 36-carbon long-chain PUFA that has previously been implicated in Japanese PDAC cohorts. This finding was confirmed in an American cohort with a 86% sensitivity and 91% specificity [75].

Composite Metabolic Signatures

Though metabolomics including lipidomics allowed the identification of clinical metabolite biomarkers, these individual metabolite signatures are far from routine clinical use, partly because of disappointing specificity when challenged to discriminate patients with PDAC from chronic pancreatitis [29, 76]. Also, the performance of these biomarkers may be affected by a significant number of variables, such as age, gender, sample collection method, duration of sample storage, and sample handling. Understanding comorbidities and how they affect the performance of biomarkers are of utmost importance [77]. These conditions may contribute to the heterogeneity in performance of candidate biomarkers [78]. Although some small-scale metabolomic studies have shown promise, the general performance of biomarkers including that of the gold standard cancer antigen 19–9 (CA19–9) in differentiating PDAC from CP is modest, and improvements are needed.

Undoubtedly, one of the greatest biomarker-related challenges in this field is finding biomarkers that accurately distinguish PDAC from other diseases of the pancreas, where overlapping signs and symptoms make differential clinical diagnosis difficult. Recently our own group reported a metabolite-based biomarker signature which distinguishes PDAC from CP with much greater accuracy than achieved by CA19–9 alone [76]. Mayerle et al. reported global analysis of 914 patients analyzed for blood (serum and plasma)-based metabolites including lipids to identify candidate metabolites that distinguish PDAC from CP. Out of 477 metabolites from 10 ontology classes, 29 metabolites were significantly altered between PDAC and CP in serum and plasma of the training set. The Elastic Net algorithm identified 9 metabolites (Table 5.2) plus CA19–9 discriminating PDAC from CP with an AUC of 0.96 [76]. This metabolomic signature was successfully validated in an independent cohort. The study was designed to accurately exclude suspected pancreatic cancer in patients with CP, with an emphasis placed on optimizing the negative predictive value (NPV) [29, 76].

In summary, the only chance of curative treatment for PDAC is based on prompt diagnosis followed by surgical treatment. Unfortunately, routine cancer markers do not seem to be reliable in prediction and detection of early stages of PDAC. Use of metabolomics-based biomarkers points to its potential for the diagnosis of PDAC and indeed for cancer diagnostics in general. The near future probably lies in a carefully selected panel of biomarkers that would allow for earlier diagnosis of PDAC and easier determination of its stage and, ideally, also allow for tailoring of treatment and to provide indicators of prognosis/outcome.

Table 5.2 List of metabolites selected based on the multivariate elastic net analysis comprising the biomarker signature

Metabolites	Ontology class	Fold changes PDAC vs CP Training data	Fold changes PDAC vs CP Test data
CA19–9	Clinical markers	18.36	14.27
Proline	Amino acids	0.69	0.75
Sphingomyelin (d18:2, C17:0)	Complex lipids, fatty acids and related	1.15	1.15
Phosphatidylcholine (C18:0, C22:6)	Complex lipids, fatty acids and related	1.26	1.06
Isocitrate	Energy metabolism and related	1.26	0.99
Sphinganine-1-phosphate (d18:0)	Complex lipids, fatty acids and related	0.79	0.85
Histidine	Amino acid	0.77	0.79
Pyruvate	Energy metabolism and related	0.93	0.97
Ceramide (d18:1, C24:0)	Complex lipids, fatty acids and related	0.79	0.80
Sphingomyelin (d17:1, C18:0)	Complex lipids, fatty acids and related	1.36	1.37

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Chapter 6
Blood-Based Circulating RNAs as Preventive, Diagnostic, Prognostic and Druggable Biomarkers for Pancreatic Ductal Adenocarcinoma

Bo Kong and Helmut Friess

Despite enormous advances in understanding pancreatic cancer biology, the prognosis of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) remains poor. This also holds for patients who undergo surgical resections [1]. Here, metastasis is the primary reason leading to cancer-related death in that 70% of patients die eventually from remote metastasis. In particular, a portion of them dies from widespread metastasis within 2 years following curative-aimed surgery [2]. Although the surgical resection is the only "curative" option for PDAC patients, the majority of patients (ca. 80% of patients) are diagnosed at the advanced and often unresectable stage. This is because PDAC with its aggressive tumour biology develops in general without specific symptoms. Thus, it is essential to develop biomarkers, which enable a risk stratification for PDAC in the general population, and eventually contributes to earlier detection. Recent studies have uncovered a stable presence of circulating RNAs in blood, which may serve as the promising biomarkers for PDAC prevention, diagnosis, prognosis and targeted therapy.

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Blood-Based Circulating RNAs

Circulating mRNA in PDAC

Blood-based circulating RNAs consist of two major categories: mRNAs (messenger RNAs) and ncRNAs (noncoding RNAs, Fig. 6.1) [3, 4]. In blood, mRNAs exist either in a cell-free or in a cellular form. Due to the abundance of RNases, cell-free mRNA can only be detected at a very low concentration in blood, and it is thought to be incorporated into exosomes or microvesicles [5]. Here, Kang and co-author reported that the serum level of type IV collagen (COL6A3) mRNA constituted potentially a diagnostic biomarker for PDAC with high sensitivity (0.91), but low specificity (0.46). As for cellular mRNA, it is mainly used as a surrogate marker for circulating tumour cells (CTCs). In 1996, Funaki and co-authors first reported the detection of carcinoembryonic antigen (CEA) mRNA in the whole blood of PDAC patients using classic RT-PCR methods [6]. Two years later, the same group reported a quantitative analysis of CEA mRNA using portal blood; they successfully detected high levels of CEA mRNAs in PDAC preoperatively, which declined significantly after tumour resection [7]. These initial data suggested that CEA mRNA in blood could be used to monitor the disease progression. Indeed, this hypothesis was tested in a study published in 2004 [8]. As such, Mataki and co-authors investigated the CEA mRNA expression in 53 patients in whole blood samples after surgical resection of biliary-pancreatic cancers. Among these 53 patients, 16 of them developed recurrence. The detection rate of blood CEA mRNA in these 16 patients was significantly higher than those without recurrence (75% vs. 5.4%, $p < 0.001$). In their analysis, the sensitivity and specificity of blood CEA mRNA were 75% and 94.6%, respectively, which is superior to conventional biomarkers such as CA19–9. More recently, the detection of cancer cell-specific blood mRNA markers such as CK20 (keratin 20) and alpha-1,4-N-acetylglucosaminyltransferase (a4GnT) was also reported [9, 10]. However, these methods were not further analysed in larger PDAC cohorts.

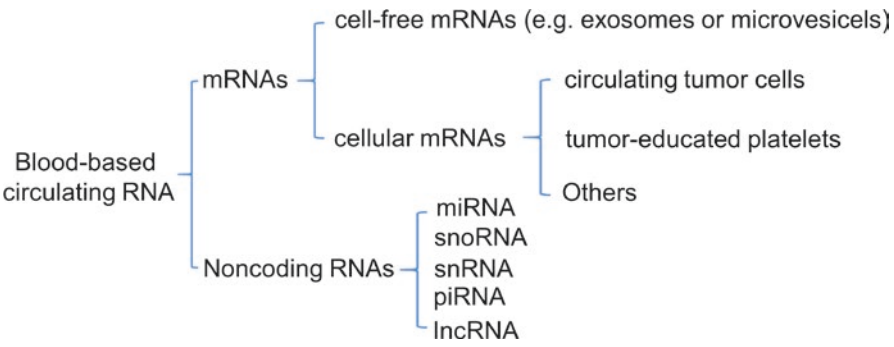

Fig. 6.1 Classification of blood RNAs

Apart from CTCs, mRNA profiles of tumour-educated platelets (TEPs) have the potential to function as diagnostic biomarkers for PDAC [11]. Taking advantage of next-generation sequencing techniques, Best and co-authors performed mRNA sequencing in 283 platelets samples including 35 PDAC patients. A panel of mRNA classifier was identified to distinguish cancer patients from healthy individuals with 96% accuracy.

Circulating ncRNAs in PDAC

It is now known that only a small part of human genomic DNAs code proteins and a large part of them are transcribed into ncRNAs [12]. ncRNAs contain miRNAs (microRNAs), small ncRNAs (nucleolar RNA (snoRNA), nuclear RNA (snRNA) and piwi-interacting RNA (piRNA)) and long ncRNAs (IncRNA). Initially, the function of these ncRNAs was thought to be merely a signal intermediate transferring genetic information from DNA to proteins. However, it became recently clear that they also played crucial roles in many cellular processes including pancreatic carcinogenesis [13].

Among these ncRNAs, miRNA is presently the most characterized one. miRNAs contain 18–22 nucleotides. They control cell proliferation, differentiation and cell death by regulating the post-transcriptional expression of genes [14]. In 2008, Mitchell and co-authors first demonstrated that miRNAs were detected in blood in a remarkably stable form protected from endogenous RNase activity [15]. Later studies revealed that circulating miRNAs are either packaged into exosome vesicles or bound to proteins in serum/plasma. Hence, they are protected from the degradation of endogenous RNase activity [16, 17]. This stable feature of miRNA in blood together with improved detection methods opens up a novel research field focusing on their potentials as noninvasive tumour diagnostic biomarkers in blood. As for PDAC, many studies have revealed that blood miRNAs might be useful for PDAC prevention, diagnosis, prognosis and therapy (Table 6.1). Also, lncRNA and snRNA were also found to be promising diagnostic biomarkers.

Table 6.1 Circulating ncRNAs as biomarkers of PDAC

	Preventive	Diagnostic	Prognostic	Druggable
Blood RNAs	miRNA	miRNA IncRNA snRNA	miRNA	miRNA
Other blood markers	BCAAs FBG	CA19–9	CA19–9	None

BCAAs branched-chain amino acids, *FBG* fasting blood glucose

Circulating ncRNAs as Preventive/Predictive Biomarkers of PDAC

Previous Blood Preventive/Predictive Biomarkers for PDAC

As for the preventive/predictive markers of PDAC, previous studies mainly focus on diabetes mellitus, which has a bi-directional connection with PDAC, with diabetes being both a risk factor and in some cases an early sign of the disease (Table 6.2) [18]. In a well-designed case-control study, fasting blood glucose (FBG) levels were increased (>126 mg/dL) in PDAC patients for a mean period of 36 to 30 months before cancer diagnosis [19]. Similarly, increased plasma levels of branched-chain amino acids (BCAAs), a marker for insulin resistance, were associated with the development of PDAC. The strongest association (risk) was observed among samples collected 24 to 60 months before cancer diagnosis [20].

Blood miRNA: "Late" Preventive/Predictive Biomarkers

As for blood miRNAs, Duell and co-authors recently published a prospective cohort study involving 225 healthy controls and 225 PDAC patients (Table 6.2) [21]. A panel of eight miRNAs (miR-10a, miR-10b, miR-21-3p, miR-21-5p, miR-30c, miR-106b, miR-155 and miR-212) was screened for their expressions in plasma samples taken before PDAC diagnosis. Four of these eight miRNAs (miR-10b, miR-21-5p, miR-30c and miR-106b) were significantly higher in PDAC plasma samples collected within 24 months before cancer diagnosis compared to healthy controls. However, compared to other preventive/predictive markers, alterations of blood miRNAs seem to take place late in the disease course of PDAC. This notion was confirmed by a recent case-control study published by Franklin and co-authors [22]. In this study, 15 miRNAs were investigated in 67 plasma samples sequentially collected before PDAC diagnosis, and their expressions were compared with 132 matched controls. However, none of these 15 miRNAs was significantly altered in prediagnostic plasma samples. In comparison, CA19–9 levels were already significantly increased in plasma samples collected less than 5 years before diagnosis. Collectively, these data suggest that alterations in blood miRNAs tend to occur late in the disease course of PDAC and blood miRNAs, as preventive/predictive early biomarkers for PDAC, are in general inferior to classical biomarkers. Thus, the current evidence does not support the notion to use blood miRNA as preventive/predictive biomarkers for patient selection in a PDAC surveillance programme.

Table 6.2 Preventive/predictive blood biomarkers of PDAC

Biomarkers	Study design	Patient number	Time before diagnosis	Year/reference
FBG	Case-control	526 PDAC	36–30 months	2018/ [19]
BCAAs	Case-control	454 PDAC 908 controls	24–60 months	2014/ [20]
miRNAs	Case-control	225 PDAC 225 controls	<24 months	2017/ [21]

Circulating ncRNAs as Diagnostic Biomarkers of PDAC

Despite its limitations, CA19–9 is the only blood biomarker that is routinely used for PDAC diagnosis [23]. Thus, it is essential to develop further blood-based biomarkers with better sensitivity and specificity especially in early tumour stage. Upon the discovery of miRNA stable presence in blood in 2008 [15], the first study exploring the diagnostic potentials of plasma miRNAs was published in 2009 [24]. In this study, 4 miRNAs, miR-21, miR-210, miR-155 and miR-196, were investigated for their expressions in 49 PDAC and 36 control plasma samples using real-time PCR. This analysis revealed that this panel of four miRNAs discriminated PDAC patients from healthy controls with a sensitivity of 64% and a specificity of 89%, respectively. However, no comparison to CA19–9 was performed in this study. Moreover, as the first "proof of principle" study, it opened a door for the clinical translation of blood miRNAs as diagnostic markers for PDAC. In the last decade, numerous studies have been published on this topic (Table 6.3). Regarding diagnostic performance, blood miRNAs tend to have comparable sensitivity, but consistently a lower specificity as compared to serum CA19–9. For instance, blood

Table 6.3 Diagnostic performance of CA19–9 and ncRNAs in PDAC

Biomarkers	Source	Patient number	AUC	Sensitivity	Specificity	Year/ reference
miR panel miR-16 miR-196a vs. CA19–9	Plasma	140 PDAC 68 controls	0.89 vs. 0.90	87% vs. 81%	73% vs. 100%	2012/ [29]
miR-1290 vs. CA19–9	Serum	41 PDAC 19 controls	0.96 vs. 0.86	88% vs. 71%	84% vs. 90%	2013/ [25]
miR panel (miR-885-5p, 22-3p,642b-3p) vs. CA19–9	Plasma	11 PDAC 11 controls	0.97 vs. Unclear	91% vs. 73%	91% vs. 100%	2014/ [27]
miR panels Panel 1 Panel 2 vs. CA19–9	Whole blood	409 PDAC 312 controls	0.80 0.91 vs. 0.81	77% 80% vs. 74%	66% 82% vs. 99%	2014/ [28]
miR-483-3p miR-21 vs. CA19–9	Plasma	32 PDAC 30 controls	0.74 0.73 vs. 0.86	Undefined	Undefined	2015/ [30]
Linc-pint vs. CA 19–9	Plasma	59 PDAC 35 controls	0.78 vs. 0.87	87% Vs. 54%	77% Vs. 82%	2016/ [32]
miR-1290 vs. CA19–9	Plasma	267 PDAC 167 controls	0.73 vs. 0.91	56.3% Vs. 85%	89.5% Vs. 95.9%	2018/ [26]

miR-1290 was found to have a sensitivity of 88% and a specificity of 84% in a small cohort consisting of 41 PDAC patients and 19 controls [25]. In this cohort, serum CA19–9 differentiated PDAC patients from controls with a sensitivity of 71% and a specificity of 90%. This trend was seen in another large series containing 267 PDAC patients and 167 controls [26]. Here, blood miR-1290 distinguished PDAC patients from healthy controls with a sensitivity of 56.3% and a specificity of 89.5%, respectively. However, this was significantly lower than a sensitivity of 85% and a specificity of 95.9% for serum CA19–9 in the same cohort. This limited specificity was also observed when panels of blood miRNAs were tested [27–29]. For example, Schultz and co-authors identified two panels of whole blood miRNAs diagnosing PDAC with a specificity of 66% and 82% in a cohort of 409 PDAC patients and 312 controls, which was also lower than 99% for CA19–9 [28]. Similarly, Liu and co-authors compared the diagnostic accuracy of a panel of plasma miRNAs (miR-16 and miR-196a) and serum CA19–9 in a cohort of 140 PDAC patients and 68 controls [30]. As compared to serum CA19–9, this panel of plasma miRNAs had a similar sensitivity (87% vs. 81%), but a lower specificity (73% vs. 100%) in differentiating PDAC patients from healthy controls. Taken together, blood miRNAs have similar sensitivity, but unfavourable specificity in detecting PDAC as compared to the routinely used serum marker CA19–9.

Apart from blood miRNAs, other blood ncRNAs such as IncRNA and snRNA were also reported to be potential diagnostic biomarkers for PDAC [31, 32]. Here, Baraniskin et al. identified fragments of circulating U2 snRNAs as a novel diagnostic marker for PDAC in a retrospective cohort [31]. Recently, Linc-pint (p53-induced transcript) was identified as a potential diagnostic lncRNA in plasma for PDAC patients [32]. Certainly, these data need to be validated by further studies with large patient numbers (e.g. prospective studies).

Circulating ncRNAs as Prognostic Biomarkers of PDAC

As earlier demonstrated, serum CA19–9 is a well-validated diagnostic marker for PDAC. Recent studies revealed that it also constituted a prognostic biomarker for PDAC patients [33, 34]. However, the cut-off value of CA19–9 as a prognostic biomarker is not routinely used “37 U/ml”, but much higher values. For instance, Dong et al. analysed the serum CA19–9 levels in a cohort of 120 PDAC patients and their prognostic impact. This analysis revealed that PDAC patients with serum CA19–9 levels less than 338 U/ml had a significantly longer median overall survival than those with serum CA19–9 above 338 U/ml (24.9 vs. 11.9 months, $p = 0.009$). The same principle also applies to blood miRNAs: many of the above-mentioned blood miRNAs with diagnostic potentials are also prognostic biomarkers when an appropriate cut-off value is applied (Table 6.4). Also, some blood miRNAs were found to be associated with other clinical factors of PDAC. For example, miR-744 is associated with the tumour size (T) [35]; miR-744 and miR-107 mainly are linked with the lymphatic status(N) [35, 36]; miR-221, miR-1290, miR-21 and miR-107 are

Table 6.4 ncRNAs as prognostic biomarkers for PDAC

Biomarkers	Patient number	Survival	Tumour size (T)	Lymphatic status (N)	Metastatic status (M)	Recurrence	Year/ reference
miR-1290 miR-486-3p	$n = 41$	+ +	– –	– –	– –	– –	2013/ [25]
miR-221	$n = 47$	–	–	–	+	–	2014/ [37]
miR-744	$n = 94$	+	+	+	–	+	2015/ [35]
miR-21	$n = 32$	+	–	–	+	–	2015/ [30]
miR-107	$n = 74$	+	–	+	+	+	2017/ [36]
miR-1290	$n = 167$	+	–	–	+	–	2018/ [26]

related to the metastatic status (M, Table 6.4); miR-744 and miR-107 are associated with tumour recurrence (Table 6.4) [37]. Thus, blood miRNAs not only affect PDAC prognosis but also are associated with multiple clinical parameters of PDAC.

Circulating ncRNAs as Druggable Targets of PDAC

Recently, miRNA-based therapy has been developed [38, 39]. For instance, miravirsen (also known as SPC3649) is a potent miR-122 inhibitor, which is currently tested for treating hepatitis C infection in humans. As for PDAC, Immaura and co-authors identified plasma miR-107 as a potentially druggable target [36]. Firstly, they observed that miR-107 was significantly down-regulated in plasmas from PDAC patients compared to healthy controls. In a xenograft mouse model of PDAC, the restoration and maintenance of plasma miR-107 using miRNA mimics significantly inhibited tumour growth. These data provided first evidence defining plasma miR-107 as a potentially druggable target in PDAC patients.

Conclusion and Outlook

Hereby, we summarized the potential utility of blood-based circulating RNAs as preventive, diagnostic, prognostic and druggable biomarkers for PDAC. Due to the abundance of RNases, the reliable detection of cell-free mRNA in blood may not be easy to realize in clinical practice, thus compromising its future application. For the cellular form of mRNAs in blood, the exact dissection of blood mRNA composition (e.g. CTCs or TEPs) is crucial for future clinical translation. As for ncRNAs, blood

miRNA is the most promising candidate. However, the alteration in blood miRNAs tends to take place late in the disease course of PDAC, arguing against its role as a preventive/predictive biomarker. Despite a lower specificity, blood miRNAs, as diagnostic biomarkers, are generally as useful as serum CA19–9 in diagnosing PDAC. The poor specificity of blood miRNAs might be improved by selectively using exosomal levels of miRNAs [40]. Furthermore, by applying appropriate cut-off values, many blood miRNAs might also serve as prognostic biomarkers useful for preoperative patient stratification. Finally, a few blood miRNAs are currently explored as druggable biomarkers at the preclinical stage.

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Chapter 7
Circulating Tumor DNA as a Novel Biomarker for Pancreatic Cancer

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Models to Derive Biomarkers

The pancreas functions as both an endocrine and an exocrine organ, with crucial roles in digestion of food and maintenance of blood glucose levels. Obstruction of pancreatic endocrine function contributes to the development of *diabetes mellitus* (DM). Alternatively, exocrine dysfunction, frequently due to *chronic pancreatitis,* causes malnutrition, and oncogene activation can lead to *pancreatic cancer*. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has a devastating prognosis despite intensive efforts in basic and translational research. PDAC has an overall 5-year survival rate of only 4%. According to recent predictions, pancreatic cancer will surpass colorectal and breast cancer to rank as the second most common cause of cancer-related deaths in Germany by 2030 [1]. The only potentially curative treatment is surgery, but only 15–20% of PDAC patients are eligible, and after surgery still just 25–30% survive. The genetic complexity and inter−/intratumoral heterogeneity of PDAC prevent the development of tailored therapies. Further, there are no predictive biomarkers that take individual tumor characteristics into account [1, 2]. The most promising way to diagnose PDAC in its curable phase would be identification of tumors at a premalignant stage, as we do with colon adenomas in the prevention of colorectal cancer [3]. The progression of PDAC begins with acinar-to-ductal metaplasia (ADM) and further development into more advanced precursor lesions called pancreatic intraepithelial

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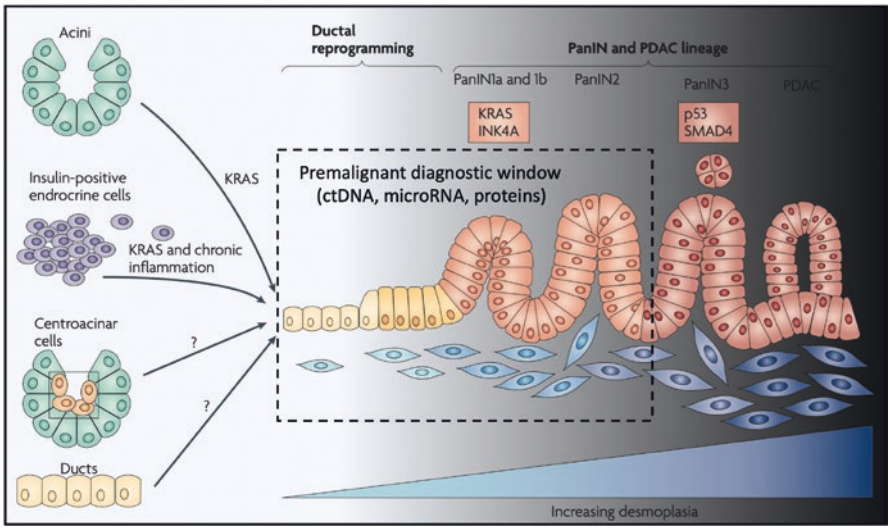

Fig. 7.1 Illustration of the sequential steps in pancreatic cancer development and the from secreted cargo arising premalignant diagnostic window. (Modified from Morris et al. [7], with permission)

neoplasia (PanIN). Hence, a biomarker to sense the development of PanIN lesions would be desirable [4]. Currently, PDAC models are limited to genetically engineered mouse models, 2D culture systems, patient-derived xenografts, and most recently pancreatic organoid cultures. The lattermost can be derived from primary cancer specimens and have been shown to be a superior model for PDAC; however, they only allow analysis of the static endpoint once the stage of early diagnosis has already passed [5]. Most biomarkers were discovered in these advanced-stage PDAC model systems that are not representative of earlier stages, when detection would be most relevant [6]. We reason that cargo such as ctDNA, proteins, or microRNA derived from precursor lesions, such as PanINs or cystic pancreatic tumors progressing to PDAC, might provide an innovative and effective opportunity to discover diagnostic biomarkers. Currently, however, there are no genetically clean, purified, and human in vitro pancreas organ culture systems, which allow spatiotemporal resolution of the secreted cargo of the developing precursor lesions of PDAC (Fig. 7.1).

ctDNA Biomarkers in the Bloodstream: Different Approaches and Technical Issues

Nowadays it is well established that cancer-specific genetic signatures are depicted in the bloodstream of cancer patients and can assist for non-invasive diagnosis, treatment monitoring under real-time conditions, and estimation of patients'

prognosis of pancreatic cancer patients. Various origins of these blood-based genetic biomarkers in pancreatic ductal adenocarcinoma (PDAC) were reported. Circulating tumor DNA (ctDNA) is considered to be the most intensively studied target for this purpose. ctDNA is released into the bloodstream in the context of active-spontaneous [8, 9] and passive secretion (apoptosis, necrosis, insufficient clearance) [10–13]. Molecular characterization of ctDNA allows non-invasive tumor-specific genotyping in malignancy [14, 15]. The exquisite biological specificity qualifies ctDNA as a promising biomarker in oncology. ctDNA is defined by the presence of (tumor-specific) mutations and is detectable in a variety of malignancies. The individual ctDNA concentrations are disease stage-dependent [16, 17]. The hype in cfDNA/ctDNA analytics is also explained by massive investments and developments in the technology sector. New digital technologies and sequencing approaches are meanwhile delivering ever higher sensitivities. Single point mutations are detectable as well as amplifications, rearrangements, and aneuploidy [18]. However, all ctDNA approaches, also the detection of minimal residual disease, require a certain degree of analytical sensitivity. Some authors demonstrated that gender, chronic inflammation, age, or tumor heterogeneity could influence the level of ctDNA [19]. The challenges can be summarized as follows:

1. The discrimination of ctDNA and physiologically occurring cfDNA in a cost-effective manner
2. The handling of extremely low concentrations of ctDNA
3. The exact quantification of the number of mutated fragments
4. Handling technical artifacts (errors) introduced during sequencing [20–23].

Recently it was reasoned that a specific enrichment of methylated DNA fragments from cfDNA could overcome the abovementioned limitations or challenges. A sensitive, immunoprecipitation-based protocol was recently published to analyze the methylome of small quantities of cfDNA. The authors even demonstrate the ability to classify early-stage cancers based on plasma cfDNA methylation patterns [24].

Furthermore, microvesicles or even exosomes, which are present in the blood of every human being, contain DNA cargo. Specifically, exosomes contain proteins and nucleic acids derived from their cell of origin. Exosomes can be isolated from blood plasma and, in addition to other markers, can be identified as tumor-specific by Glypican-1 on their surface [25]. Generally, the DNA contained in exosomes (exoDNA) is present in larger fragments of more than 10 kKB and is protected against degradation [26, 27]. Recently, it was shown that treatment with engineered exosomes (called iExosomes) facilitates for direct and specific targeting of oncogenic *KRAS* in pancreatic tumors and subsequently can delay tumor growth [28]. Thus, both types of DNA, ctDNA and exoDNA, can provide access to the molecular signature of the respective tumor by means of a simple blood sample; although the technical requirements for both approaches are very different, both demand a minutely developed methodological setup.

Recent work on material from patients with different types of tumors has shown that ctDNA maps the mutational makeup of a given tumor and may therefore be

used to reconstruct the latter and subsequently to monitor tumor evolution during therapy [16, 29, 30]. However, most of these works only show the feasibility based on a single analytical time point. For example, in cystic premalignant lesions of the pancreas, it was demonstrated that a diagnosis can be made by molecular characterization of ctDNA from blood plasma using highly sensitive methods (digital PCR) [4]. Based on this, mutational signatures in ctDNA were investigated in the course of therapy. Studies showed that in patients with colon cancer, the detection of *KRAS* mutation status in ctDNA and the determination of allelic frequencies in the further course allow non-invasive imaging of tumor burden and disease progression. In subgroups, a correlation of cfDNA amount under treatment with the duration of progression-free survival (PFS) could be presented [31]. Other work has shown that the seven most frequently mutated genes in PDAC are depicted in ctDNA, assessed by targeted deep sequencing, delivering comparable results with tissue-derived DNA. Such panel or digital PCR-based approaches operate generally fast, are efficient, and are rather easy to be elaborated; however, despite these promising results, small gene panels are overall not insufficient to map the entire heterogeneity. In addition, the complexity of biological processes such as clonal evolution of pancreatic carcinoma, in all cases, to completely trace the clinical course [32]. However, this is essential if therapeutic decisions based on molecular ctDNA characterization should be made. Here, whole-exome sequencing from ctDNA samples can step in but deliver the appropriate results at expensive of feasibility and pricing.

ctDNA as Biomarker for Premalignant Pancreatic Cystic Tumors and Early PDAC Progression

Earlier detection is the key to reduce cancer-related deaths. Based on published results, it appears realistic that early detection can become possible by simple blood tests [33] operating on ctDNA analytical basis [34]. PDAC is the most common malignant tumor of the pancreas and the fourth leading cause of cancer deaths in the western world with an increasing incidence [35]. Overall, PDAC has a very poor prognosis despite intensive treatment regimens [36, 37]. Therefore, all efforts for prevention and for early detection of pancreatic malignancy must be made. A promising approach in this regard is the non-invasive monitoring of PDAC precursor lesions using liquid biopsy approaches, to avoid the malignant transformation of precursor lesions. In that sense, it is necessary to define high-risk lesions in the pancreas, which are known to develop into PDAC. These include primarily cystic pancreatic neoplasms, such as mucinous cystic neoplasms (MCN) or intraductal papillary-mucinous cystic neoplasm (IPMN) [38, 39]. Consequently, correct management of cystic pancreatic tumors may prevent progression to PDAC while minimizing the need for lifelong screening and related costs [40]. The diagnosis and monitoring of these lesions has so far invariably been based on instrumental

examinations. In a retrospective analysis, it was shown that the genetic profile of cystic pancreatic tumors is depicted in the blood and is usable for, e.g., diagnostics in the form of a liquid biopsy. Indeed, *GNAS* and *KRAS* mutations in ctDNA significantly discriminated patients with strictly benign pancreatic lesions (serous cystadenomas) from others with borderline cysts (IPMN) or pancreatic cancer [40].

Obviously, proteins released from precursor lesions during tumor progression such as pancreatic intraepithelial neoplasia (PanIN) might serve also as innovative and effective diagnostic biomarkers. In turn, a future path to succeed in early diagnosis of PDAC might be the combination and complementary use of established markers such as CA19-9 together with novel protein and cfDNA-based approaches. Thrombospondin-2 (THBS2) is a disulfide-linked homotrimer glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. THBS2 probably inhibits angiogenesis, and depletion of the THBS2 gene in a mouse model increases the susceptibility to cancer [41]. THBS2 is secreted or released from human precursor PanIN organoids and may hence serve as a biomarker for early PDAC [41, 42]. Preclinical data were recently validated in a large cohort with PDAC patients at various disease stages compared to healthy controls and patients with cystic tumors or chronic pancreatitis. Normal pancreatic cells express the THBS2 antigen, but under physiological conditions, the plasma concentration is low. In contrary, it is highly expressed by PDAC tumor cells, and the plasma of PDAC patients shows elevated THBS2 levels. The concentration of THBS2 in plasma is reported to allow the discrimination between resectable PDAC stage I cancer and advanced stage III/IV. However, the mechanism of THBS2 release into the bloodstream remains elusive [43]. The value of THBS2 by complementing with cfDNA measurements and CA19-9 in a large cohort of PDAC patients prior to intended curative surgery was evaluated and compared to strictly benign IPMN patients and healthy controls. The authors reported that the combination of CA19-9 and THBS2 showed a promising c-statistics of 0.87 and could be further increased to 0.94 when combining CA19-9, THBS2, and total cfDNA quantification. This marker combination performed best for all PDAC stages, especially in the group of stage I PDAC (c-statistics of 0.90 for the three-marker combination) [44].

Recent studies have shown that dynamic changes in the global DNA methylation and gene expression patterns play key roles in the PDAC development, which was supported by integrated genomic analysis of hundreds of PDAC cases which allows to define distinct molecular subtype of PDAC [45]. Differential methylation is observed in genes associated, for example, with pancreatic development and pancreatic cancer core signaling pathways [46]. First data are now available that epigenetic targeting might be a new therapeutic option in PDAC [47]. The sensitivity of ctDNA mutagenome analyzing methods may be low among patients with early-stage cancer given the limited number of recurrent mutations [33, 34, 48, 49]. Shen SY et al. assumed that large-scale epigenetic alterations potentially have greater ability to detect and classify cancers in patients with early-stage disease and developed a sensitive blood test by using plasma cfDNA methylomes [24].

Prognostic Relevance of ctDNA Signatures During Treatment of Resectable PDAC

Liquid biopsy approaches also were studied in the context of resectable PDAC as a prognostic biomarker. A French study by Pietrasz D et al. could show that patients, resected from PDAC, with undetectable ctDNA after surgery had a longer disease-free survival (17.6 vs. 4.6 months; log-rank $P = 0.03$) and a longer overall survival (32.2 vs. 19.3; $P = 0.027$) than those with detectable ctDNA, based on genotyping of ctDNA for frequent mutations such as in *CDKN2A*, *SMAD4*, *TP53*, or *KRAS* [50]. In patients with advanced PDACs, ctDNA was also an independent prognostic biomarker for survival (HR = 1.94; $P = 0.007$). Chen and colleagues have previously described the prognostic value of ctDNA as a biomarker in PDAC [51]. In their series, the presence of *KRAS* mutation in plasma was correlated with poor OS (3.9 vs. 10.2 months; $P < 0.001$) in nonresectable patients. More recently, Sausen and colleagues reported that, in resectable patients, ctDNA was a prognostic factor of early tumor relapse if detected before surgery (log-rank $P = 0.015$). In this study, in a subgroup of 20 patients collected after surgical resection, detectable ctDNA was also a prognostic biomarker of DFS (9.9 months vs. median not reached; log-rank $P = 0.02$). Taken together, the detection of ctDNA after resection predicts clinical relapse and poor outcome, with recurrence by ctDNA detected 6.5 months earlier than with CT imaging. These observations provide genetic predictors of outcome in pancreatic cancer and have implications for new avenues of therapeutic intervention [52]. Hadano and colleagues reported that among 105 PDAC cases, ctDNA was detected in 33 (31%) plasma samples. The median OS durations were 13.6 months for patients with ctDNA (ctDNA+) and 27.6 months for patients without ctDNA. Patients who were ctDNA+ had a significantly poorer prognosis with respect to OS ($P < 0.0001$) [53]. The combination of ctDNA and exoDNA analyses in PDAC was recently published. Bernard and colleagues performed a prospective cohort study and collected liquid biopsy samples 34 resectable PDAC patients. Droplet digital polymerase chain reaction was used to determine *KRAS* mutant allele fraction (MAF) from ctDNA and exoDNA purified from plasma and was correlated with prognostic and predictive outcomes [54]. Interestingly, an increase in exoDNA level after neoadjuvant therapy was significantly associated with disease progression ($P = 0.003$), whereas ctDNA did not show correlations with outcomes [54].

ctDNA in Metastatic PDAC: Prognostics and Real-Time Treatment Guidance

The first studies on ctDNA in PDAC focused on *KRAS* mutations that are present in the majority of PDACs [52, 55, 56]. But for treatment-associated tumor evolution, more genomic alterations are likely to play a role. Currently, there are only limited

data available from ctDNA analyses over and above *KRAS* profiling [50, 57]. Pietrasz and colleagues reported that 64.7% of the patients with metastatic PDAC had detectable ctDNA in comparison with only 16.6% with locally advanced disease ($P < 0.001$ [50]). In the group of metastatic patients, no significant correlation was found between the presence of ctDNA and the number of metastatic sites ($P = 0.13$). The presence of ctDNA was strongly correlated with poor OS (6.5 vs. 19.0 months; log-rank $P < 0.001$) in patients with advanced pancreatic adenocarcinoma. Patients with higher MAF had the worst OS. The OS decreased from 18.9, 7.8, and 4.9 months (log-rank $P < 0.001$) for the lowest, middle, and highest MAF tertiles, respectively [50].

In addition, a recently published study applied a targeted next-generation sequencing approach of ctDNA, combined with droplet digital PCR, (i) to examine ctDNA as a tool for non-invasive diagnosis and (ii) to inform on therapy-induced tumor evolution in metastatic PDAC during different lines of systemic treatment [32]. All therapy-naïve patients presented with detectable ctDNA at baseline. The combined mutational allele frequency (CMAF) of *KRAS* and *TP53* was reported to reflect the amount of ctDNA. The median CMAF level significantly decreased during treatment ($P = 0.0027$) and increased at progression ($P = 0.0104$). CA19-9 tumor marker analyses did not show significant differences. In treatment-naïve patients, the CMAF levels during therapy significantly correlated with progression-free survival (Spearman, $r = -0.8609$, $P = 0.0013$) [32].

Kruger and colleagues stated in a previously published study that repeated ctDNA measurements on mutated *KRAS* alleles represent a novel and promising tool for early response prediction and therapy monitoring in advanced pancreatic cancer [58]. The authors reported that mut*KRAS* ctDNA was present in a majority of advanced PDAC patients (67%). The presence of mut*KRAS* ctDNA was significantly correlated to an adverse overall survival. A decrease in mut*KRAS* ctDNA levels during therapy was an early indicator of response to therapy, while there was no significant correlation between kinetics of CA19-9 tumor marker [58].

Summary and Conclusion

ctDNA-based measurements have the capacity to relaunch the biomarker debate in pancreatic cancer. The reason for this ascent is multilayered but primarily links a novel grade of specificity due to the opportunity to detect tumor-specific alterations with an unreached sensitivity resulting from technological progress in this field. The latter can be particularly interesting when a PDAC needs to be differentiated from its yet benign precursor lesions or to risk stratify a cystic tumor in the pancreas. A further additive value is given by complementary action with established biomarkers such as CA19-9. Besides the diagnostic value of ctDNA measures, the most important strength lies in its capacity to mimic the entire mutational makeup and thus PDAC's heterogeneity plus the opportunity to quantitatively follow the mutational load to track and trace chemotherapy-driven tumor evolution. In that light, a

therapeutic blueprint based on repetitive ctDNA genotyping can be envisioned to specifically tailor patients' therapy. Still, clinical grade standards and future validation of this novel tool need to developed, and economical hurdles to bring this method to a broader range have to be negotiated.

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Chapter 8
PDAC Subtypes/Stratification

Holly Brunton, Giuseppina Caligiuri, Gareth J. Inman, and Peter Bailey

Large-scale sequencing analyses have transformed our understanding of pancreatic ductal adenocarcinoma (PDAC) and have defined several molecular taxonomies that now guide pre-clinical and clinical therapeutic development. The identification of molecularly defined subgroups of patients with distinct biological underpinnings and potential therapeutic vulnerabilities promises a step change in clinical practice. However, the ability of these molecular taxonomies to guide therapy and ultimately improve patient outcomes remains to be established. This review examines the current status of molecular subtyping in PDAC and explores subtype-specific biology, potential subtype-specific vulnerabilities and their potential relevance to clinical practice.

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From Single Genetic Aberrations to Actionable Genomic Subtypes

International sequencing consortia have molecularly profiled over 25,000 genomes [1, 2]. At the outset, these studies promised to transform clinical decision-making by identifying genetic aberrations or actionable mutations in individual patients, such as recurrent hot-spot mutations in oncogenes, that are susceptible to therapeutic intervention. To date, however, this promise has only been realised in a relatively small number of cancer types with recurrent BCR-ABL gene fusions in chronic myeloid leukaemia (CML) being a prime example of a recurrent actionable mutation (targetable by tyrosine kinase inhibitors) that has transformed clinical practice and patient outcomes [3, 4].

PDAC is one cancer type where the utility of patient selection, based on the presence of a single actionable mutation, is severely challenged by a paucity of recurrent clinically actionable events [5–12]. The PDAC mutational landscape is dominated by recurrent, predominantly overlapping mutations in KRAS, TP53, SMAD4 and CDKN2A (>50%) with a subset of additional genes including KDM6A, MLL3, ARID1A, TGFBR2, RBM10 and BCORL1 recurrently mutated in 5–10% of patient samples [6, 10]. The mutational landscape of PDAC is, however, complex with a long tail of low prevalence mutations contributing to significant intra-tumour heterogeneity. The majority of single gene aberrations occur at low prevalence (<2%) with genetic biomarkers of drug response such as ERBB2 amplification, BRAF gene fusions/mutations and BRCA1/2 falling within this long tail of low prevalence mutations [6, 13, 14]. Despite the obvious relevance of these predictive biomarkers to clinical practice, their low prevalence in PDAC patient populations has limited their uptake as economically viable therapeutic targets.

Notwithstanding the inherent challenges in defining patient groups using single genetic biomarkers of therapeutic response, other readouts of genomic abnormality including structural variation (SV) and mutational signatures have defined larger patient subgroups with potential clinical utility [6, 15]. SVs including deletions, amplifications, duplications and translocations can be grouped on the basis of frequency and distribution to define four genomic SV subtypes, namely, stable (<50 structural variations per genome); scattered (50–200 structural variants per genome); locally rearranged (>200 structural variants clustered on less than 3 chromosomes); or unstable (>200 structural variants distributed across the genome). Of these, the unstable SV subtype is significantly associated with subgroups of patients having mutations in DNA damage repair pathway (DDR) genes including BRCA1, BRCA2 and PALB2 [6]. Six mutational signatures, which define specific mutational processes active in tumour cells, have been identified in PDAC. Four of these mutational signatures are associated with known mutational processes and include a BRCA mutational signature, an age-related signature, a DNA mismatch repair (MMR) deficiency signature and an APOBEC signature (APOBEC family of cytidine deaminases) [6, 15].

The identification of subgroups of patients harbouring genomic abnormalities that are associated with defects in DNA damage repair and/or MMR highlights the

potential utility of these genomic readouts for clinical decision-making. A hallmark of cancers with defective DDR is their vulnerability to specific DNA damaging agents such as platinum and PARP inhibitors. Platinum-based therapies are widely used in other cancer settings, and there is growing evidence for their efficacy in PDAC [6, 16]. Exceptional responders to platinum therapy are well documented in small subsets of PDAC patients, and BRCA1 and BRCA2 germline carriers show significant responses to both platinum and PARP inhibitors [6]. Although germline and somatic mutations in DDR pathway genes such as BRCA1/2 occur at low prevalence, the integration of orthogonal genomic readouts of DDR deficiency suggests that platinum therapy and/or novel drugs targeting similar mechanisms (such as PARP inhibitors) may be effective in a large subset of PDAC patients [6]. It has been calculated that 24% of all PDAC tumours harbour either an unstable genomes (>200 structural variants per genome); somatic and germline mutations in BRCA pathway genes; a BRCA mutational signature; or combinations thereof [6]. Importantly, the classification of PDAC patients using a combination of these orthogonal measures can predict response to platinum therapy [6].

Microsatellite instability (MSI) occurs in 1–2% of resectable PDAC and is a hallmark of DNA mismatch repair deficiency which is commonly associated with mutations in the MMR genes MSH2 and MLH1 [7]. MSI is reliably detectable using immunohistochemical assays for MSH1, PMS2, MLH1 and MSH6 expression [7] or NGS (single gene mutations and MMR mutational signature) [6, 17] and is a predictive biomarker of response to immune checkpoint inhibitors [18]. Pembrolizumab, which selectively targets the lymphocyte programmed cell death 1 receptor (PD-1), has recently been approved as a first-line treatment for solid tumours with MSI [19]. Recent evidence also suggests that ARID1A plays a role in DNA mismatch repair with deleterious mutations in ARID1A associated with increased immune infiltrates and significant antitumor response to immune checkpoint inhibitors [20]. These findings suggest that the combination of both MSI and deleterious ARID1A mutations may define a larger group of PDAC patients responsive to immune checkpoint inhibitors but this remains to be determined. In addition, ARID1A mutations have been shown to induce an increased reliance on ATR as a consequence of topoisomerase 2A and cell cycle defects and are consequently more sensitive to ATR inhibitors [21]. This study highlights the potential of using ARID1A mutational status as a readout for ATR targeting and provides further evidence that ARID1A mutational status may be an important biomarker of therapeutic response in PDAC.

A key challenge in deploying mutational profiling in the clinic is defining which genomic events in a given tumour are “actionable” [22]. In particular, although platforms and methodologies to detect mutations and/or genomic abnormalities are proceeding at pace, our ability to understand the relevance of these aberrations with respect to clinical decision-making remains a significant challenge. Further, despite some success in defining DDR deficiency as a large actionable segment in PDAC (approx. <= 24% of patient cohorts), additional biomarkers of therapeutic response are urgently required. In this regard, the transcriptomic profiling of PDAC has defined additional patient subgroups with potential therapeutic vulnerabilities and is helping to redefine our understanding of PDAC tumour biology [10, 12, 23–26].

Transcriptomic Subtypes of PDAC

The identification of intrinsic subtypes using gene expression data has been successfully employed in a number of different cancer settings to define broad and potentially actionable subgroups of patients. As an exemplar, the classification of colorectal cancer by gene expression profiling has identified robust and reproducible subtypes that show promise in clinical practice [27–30]. mRNA profiling of PDAC by several different groups has defined *at least* four intrinsic molecular subtypes and produced three major classification schemes with differing nomenclature [10, 12, 23–26]. A comparison of these schemes highlights several important similarities and dichotomies and underlines the need to align efforts to generate a new consensus classification for PDAC that better defines patient subgroups and clinical decision-making.

Three major studies, in particular, have shaped debate concerning the classification of PDAC using gene expression profiles. The first of these, performed by *Collisson and Sadanandam* et al., used primary resected PDAC (micro-dissected to remove stromal contamination) to define three subtypes referred to as exocrine-like, classical and quasi-mesenchymal [23]. Genes associated with exocrine function (digestive enzyme genes), markers of epithelial adhesion and terminal differentiation (e.g. GATA6) and gain in mesenchymal function were specifically expressed in either the exocrine-like, classical or quasi-mesenchymal subtypes, respectively. In addition, the quasi-mesenchymal subtype was correlated with high tumour grade and poor patient outcomes.

The second major study performed by Moffit et al. used a supervised classification approach to informatically segregate tumour cell *intrinsic* gene expression signatures from "contaminating" gene expression signatures commonly associated with terminally differentiated normal pancreas (exocrine and endocrine genes signatures) and stromal cell populations (pancreatic stellate gene signatures) [24]. This analysis identified two major PDAC tumour cell *intrinsic* subtypes named classical and basal-like and additional tumour cell *extrinsic* or stromal subtypes referred to as normal and activated. Importantly, this study was the first to model the complex interplay between tumour cell intrinsic subtypes and specific stromal cell signals with combinations of tumour-specific and stromal subtypes associated with different patient survival.

The third major classification scheme proposed by Bailey et al. used primary resectable PDAC with >40% cellularity to define four subtypes referred to as aberrantly differentiated endocrine exocrine (ADEX), pancreatic progenitor, immunogenic and squamous [31]. These subtypes overlapped directly with the Collisson classification Scheme [23] with the exception of the immunogenic subtype which was defined by the significant enrichment of genes associated with specific immune cell populations, including T cells and B cells. Although gene expression values defining the immunogenic subtype most certainly originate from immune infiltrates resident in the tumour stroma, an underlying pancreatic progenitor-like gene expression profile was clearly evident in tumours falling within this subtype. In addition,

the quasi-mesenchymal subtype of Collisson was renamed squamous due to the significant enrichment of several pan-squamous characteristics, including mutations in KDM6A, enrichment of the ΔNTP63 isoform of p63 and a significant association with adenosquamous PDAC histology. This study also demonstrated for the first time that squamous tumours undergo a profound epigenetic shift, with changes in DNA methylation orchestrating the downregulation of pancreatic specific transcription factors (PDX1, GATA6, HNF1A), which control pancreatic cell fate determination, and the activation of multigene programmes regulated by ΔNTP63 and c-MYC that drive squamous-like differentiation. Supporting a role for epigenetic dysregulation in the genesis of PDAC subtypes, the squamous subtype was found to be enriched for mutations in COMPASS (COMplex of Proteins Associated with Set1-like) complex members KDM6A, MLL2 and MLL3 that function as chromatin-modifying enzymes.

Recent studies performed by Puleo et al. [26] and Maurer et al. [32] have started to refine our understanding of these established classification schemes and in particular describe in greater detail how different stromal cell populations exist in concert with tumour cell intrinsic subtypes. Puleo et al. transcriptomically profiled 309 resected PDAC tumours to define 5 subtypes using both tumour cell intrinsic and microenvironment-derived expression signatures. This work identified two subtypes with low stromal content referred to as pure basal-like and pure classical and three additional subtypes with high stromal content referred to as stroma activated, desmoplastic and immune classical. In a complementary study, Maurer et al. performed laser capture microdissection on resected PDAC to transcriptomically profile pure epithelial or stromal cell populations. This analysis identified two major stromal subtypes, an extracellular matrix-rich (ECM-rich) and immune-rich subtype, with basal-like tumours exhibiting an ECM profile having the worst overall survival. Importantly, both Puleo et al. and Maurer et al. provide evidence that the previously proposed exocrine-like/ADEX subtype is not a genuine PDAC subtype but rather a consequence of normal pancreatic contamination in profiled tumour samples.

Towards a Consensus Transcriptomic Classification of PDAC

The transcriptomic classification of PDAC by several different groups has generated a number of interesting contrasts and ultimately divided opinion. A major point of difference concerns the inclusion of the exocrine-like/ADEX subtype as a bona fide subtype of disease. The weight of current opinion is now favouring the exclusion of this subtype on the basis that it represents normal pancreatic contamination [12, 24, 26, 32]; however, the identification of exocrine-like/ADEX gene expression in patient-derived xenografts and cell lines suggests that further study is required [8, 33, 34]. A second point of contention concerns the inclusion of a separate immunogenic subtype. Bailey et al. demonstrate that the immunogenic subtype is a complex admixture of gene expression comprising both pancreatic progenitor-like and

immune gene expression (predominantly associated with T cells and B cells) [10]. The separation of the pancreatic progenitor signature into immune high (Immunogenic) and immune low (pancreatic progenitor) suggests that signals from the underlying epithelium (immunogenic subset) may drive tumour cell immunogenicity. Recent studies, however, argue that immune infiltrates are enriched across all tumour intrinsic subtypes and their prevalence is primarily driven by tumour cellularity of sequenced samples [12]. In addition, these studies advocate the use of integrated classification schemes that apply both tumour cell intrinsic and stromal subtype signatures to optimally define prognostic PDAC subtypes [26, 32].

Despite differences in nomenclature and interpretation, a direct "side-by-side" comparison of the established classification schemes demonstrates considerable overlap and several common themes. In particular, strong alignment exists between the classical-pancreatic progenitor and quasi-mesenchymal/basal-like/squamous subtypes. Together these overlapping subtypes define two broad prognostic classes (referred to herein as classical-pancreatic and squamous) with squamous tumours associated with significantly poorer outcomes. These classes are delineated by the differential expression of pancreatic specific transcription factors, such as GATA6, PDX1 and HNF1A, that act to specify and maintain pancreatic identity and which are lost in squamous tumours. Importantly, the dynamic changes in gene expression observed between the classical-pancreatic and squamous classes are driven by an underlying shift in the epigenome. Multiple studies have now established that the squamous subtype is defined by changes in DNA methylation that ultimately repress pancreatic identity and activate multigene programmes that drive squamous-like differentiation [12, 26, 35]. Further, despite different approaches in modelling stromal infiltrate and the ever-growing number of stromal subtypes, there is a clear consensus that signals from the stroma play an important role in disease progression. An outstanding question in this regard is whether tumour cell intrinsic subtypes contribute to the levels and/or composition of stromal (fibroblasts and immune cell) infiltrate. Additional refinement and integration of tumour cell intrinsic and stromal subtype signatures will help to drive a greater understanding of tumour-stroma crosstalk and ultimately inform better prognostic models of disease.

Pre-clinical Models, Transcriptomic Subtypes and Subtype Plasticity

The LSL-Kras$^{G12D/+}$, LSL-Trp53$^{R172H/+}$, Pdx-1-Cre (KPC) genetically engineered mouse model (GEMM) is the standard model for understanding PDAC and recapitulates many of the key characteristics of human disease including the formation of precursor lesions (PanINs) leading to frank PDAC and the development of metastatic lesions within distant organs including the liver [36, 37]. The comprehensive interrogation of KPC GEMMs has identified distinct cellular populations important for disease progression and in particular has highlighted an important role for

stromal cells including *c*ancer-*a*ssociated *f*ibroblasts (CAFs) and immune cells in this process [38, 39]. Importantly, recent work has demonstrated that epithelial-derived cells isolated from KPC tumours recapitulate the transcriptionally defined subtypes of human PDAC [40]. Consistent with human disease, murine tumours having squamous transcriptional profiles are associated with high-grade poorly differentiated histologies, whereas tumours having classical-progenitor gene expression profiles are associated with low-grade well-differentiated histologies. This work also demonstrates that these histological and transcriptionally distinguishable PDAC subtypes exhibit distinct modes of migration.

Recent evidence demonstrates that stromal cues play an important role in modulating tumour cell intrinsic subtypes [39, 41, 42]. Extensive desmoplasia is a hallmark of PDAC and is characterised by a dense fibrotic stroma comprising CAFs and immune cells. A complex cocktail of tumour cell intrinsic and stromal cues help to shape this tumour microenvironment (TME) with signals from both CAFs and specific immune cell populations directing the differentiation state of PC tumour cells. In particular, PDAC exhibits substantial immune cell heterogeneity, and there is now a growing appreciation that tumour cell intrinsic factors shape the immune TME [10, 39, 42, 43]. PDAC subtypes are associated with distinct immune cell populations with tumours exhibiting a classical-pancreatic subtype enriched for transcripts associated with B cells, CD4+ and CD8+ tumour-infiltrating lymphocytes (TILs) and tumours falling within the squamous subtype characterised by myeloid cell gene enrichment and a general absence of B-cell and T-cell transcripts [10]. Remarkably, targeted ablation of myeloid cells in KPC GEMMs by the selective inhibition of CSF1R produces a profound shift in subtype from predominantly squamous-like to classical-pancreatic [42]. Inhibition of CSF1R causes a profound reprogramming of the tumour cell intrinsic pathways underpinning PDAC subtypes, including the re-activation of transcriptional networks controlled by transcription factors that act as master regulators of exocrine or endocrine pancreatic identity. Further underpinning an important paracrine role for the stroma in PC, signalling cues originating from CAFs have also been shown to modulate tumour cell intrinsic pathways [41]. Specifically, stromal cues have been shown to drive distinct changes in tumour cell metabolic pathways and to re-programme the tumour epigenome [41].

Recent evidence also demonstrates that the two major transcriptomic subtypes of PDAC are defined by distinct epigenetic landscapes that are largely shaped by specific subsets of TFs that orchestrate subtype-specific multigene programmes [10, 35, 44]. Using a collection of 23 PDAC patient-derived tumour xenografts, Lomberk et al. used chromatin states to epigenetically classify PDAC subtypes [35]. Three major epigenetic states were established described as cluster 1 (composed of enhancers active in most squamous samples), cluster 2 (enhancers active in classical-pancreatic samples) and cluster 3 (active promoters in classical-pancreatic samples). TFs associated with super-enhancers in the classical-pancreatic subtype included GATA6, FOS, FOXP1, FOXP4, KLF4, ELF3 and CUX1. Squamous-specific super-enhancer regulation was associated with the hepatocyte growth factor receptor MET. Interestingly, MET siRNA-mediated knockdown in squamous samples induced a transcriptional switch towards classical-pancreatic associated gene

programmes, in particular those driven by GATA6. This evidence implicates super-enhancers as critical regulatory hubs that both maintain pancreatic identity and control the activation of genes that drive squamous differentiation. Additionally, this data demonstrates that certain subtype-specific gene programmes maintain a degree of plasticity that can be manipulated therapeutically.

Dysregulation of super-enhancer activity by inactivation of key chromatin modifiers including KDM6A may lead to a loss of both pancreatic identity and the activation of squamous gene programmes. Consistent with this hypothesis, squamous tumours are enriched for mutations in COMPASS-like complex members KDM6A, MLL2 and MLL3. These findings suggest that mutations in key chromatin effectors may rewire the regulatory landscape of PDAC and subvert cell fate decisions to favour squamous-like cell states. In support of this notion, GEMMs of PDAC with targeted deletion of Kdm6a in the context of oncogenic Kras develop squamous-like metastatic pancreatic cancer that phenocopies the progression and histological features of human disease [45]. Mechanistically, deregulation of the COMPASS complex by Kdm6a deletion induces the aberrant activation of super-enhancers regulating the expression of ΔNTP63, MYC and RUNX3 that in turn subvert pancreatic identity and induce squamous differentiation. In a complementary study, the overexpression of ΔNTP63 was shown to drive a classical-pancreatic to squamous transcriptional reprogramming in human classical-pancreatic PDAC cells [46]. As found in the mouse Kdm6a GEMM, squamous identity was associated with profound alterations in enhancer landscape.

The plasticity exhibited by PDAC cells has important implications for disease progression, drug resistance and the development of subtype-specific therapies. Deciphering the transcriptional regulatory networks underpinning subtype plasticity will provide important mechanistic insights into disease progression and highlight potential therapeutic vulnerabilities.

PDAC Subtyping and Translational Protocols

The translation of molecular subtypes into clinical practice is in its infancy; however, several groups have made significant gains in applying genomic and/or transcriptomic subtyping to inform patient selection for targeted therapy. To bridge the translational gap between molecular subtyping and clinical decision-making, the PancSeq protocol was developed which enables rapid turnaround genomic analysis of metastatic or locally advanced PDAC [47]. Mutational signature analysis of WES data identified four main mutational signatures described as COSMIC 1 (C > T transitions at CpG dinucleotides, Aging), COSMIC2 and 13 (APOBEC), COSMIC3 (HRD and BRCA deficient) and COSMIC17 (unknown) which converged on at least two well-established subtypes of PDAC including a classical-pancreatic subtype and a squamous subtype. Interestingly integrated analysis which included

normal tissue gene expression as well as that of tumours was able to identify not only samples by subtype but also site of biopsy, suggesting that different tumour locations have differing tumour biology. In this cohort of 71 patients, 37% harboured germline or somatic mutations in DDR genes, 9 of whom were characterised as having an enrichment for the HRD/COSMIC3 signature. A further 7% of patients also had enrichment for the HRD/COSMIC3 signature but no apparent HR gene mutations. Two of these patients could be explained by downregulation of the mRNA of the HR repair protein RAD51C, highlighting the importance of using multiple methods of omics characterisation to obtain the full spectrum of potential therapeutic candidates. Furthermore, integration of the unstable SV subtype with the HRD/COSMIC3 mutation signature and DDR gene mutations could further identify potential responders to DDR therapy.

Using genomics-driven precision medicine, Aung et al. demonstrate the feasibility of using whole genome and RNA sequencing within a clinically relevant timeframe to direct clinical decision-making and identify individuals predicted to be sensitive to chemotherapy [48]. The COMPASS (Comprehensive Molecular Characterization of Advanced Pancreatic Ductal Adenocarcinoma for Better Treatment Selection) trial identified that PDAC patients with stage III/IV and transcriptionally subtyped as classical-pancreatic responded better to first-line chemotherapy compared to squamous tumours, demonstrating that better or exceptional responders could be identified using subtyping methodology.

Recent success to map clinical response with transcriptomic subtypes has been observed using a pancreatic cancer patient-derived organoid (PDO) library [49]. The PDO library is composed of 66 PDO cultures obtained from primary tumours and metastases that recapitulates the transcriptional classical-pancreatic and squamous subtypes and the mutational landscape of primary pancreatic cancer. Within the library, 57 of these organoids were isolated from 55 treatment-naïve patients, which offers a unique research tool to establish the transcriptional landscape before neoadjuvant therapy that typically occurs before surgical resection. Tiriac and colleagues demonstrate that within a clinically meaningful timeframe, drug-sensitivity profiles can be generated that reflect a patient's response to therapy. Therapeutic profiling which was termed "pharmacotyping" was performed on the PDAC PDOs using commonly used chemotherapeutics used to treat PDAC, and for each chemotherapeutic agent, the PDO library was subtyped into three groups: the least responsive, the most responsive and those exhibiting intermediate response. Gene expression signatures were further refined to include genes whose expression correlated with drug sensitivity. When the gemcitabine-specific PDO-sensitive signature was applied to a subgroup of patients who received gemcitabine monotherapy, patients with significantly better PFS were found to be enriched for the gemcitabine-sensitive signature. Importantly, the same analysis on treatment-naïve patients failed to identify individuals with improved PFS or OS suggesting that this signature is treatment dependent and may be clinically relevant for predicting response to and ultimately selection of patients for gemcitabine treatment.

Conclusions

Pancreatic cancer is associated with dismal patient outcomes. Most patients are unsuitable for surgical resection, and current treatment regimens have not required routine molecular profiling. Consequently, most patients receive non-targeted and unselected combination chemotherapy. Recent comprehensive molecular landscaping studies on samples obtained from fine needle biopsies, surgical biopsy and autopsy coupled with profiling of patient-derived cell lines and organoids are beginning to reveal a potentially brighter future for PC management. Integrated genomic and transcriptomic analyses have enabled the generation of molecular signatures that reveal the underlying biology of PC, identify potential therapeutic vulnerabilities and may predict patient response to chemotherapy. The robustness of these signatures will only increase with the inclusion of more samples, the development of sequencing methodologies and integration of pre-clinical, clinical and clinical trial-associated datasets. Increasing the breadth and depth of our datasets will enable the use of artificial intelligence and deep learning approaches to generate more clinically meaningful classifiers. Ultimately, we hope that these studies will enable the development of molecularly based and cost-effective companion diagnostics that inform clinical decisions that result in improved patient outcomes.

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Chapter 9
Circulating Tumor Cells as Biomarkers in Pancreatic Cancer

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Pancreatic cancer is highly lethal; the majority of patients present with metastatic disease at diagnosis, precluding surgical resection, which remains the only possibility for a cure in most cases [1]. Moreover, in those patients with clinically localized disease who undergo potentially curative surgical resection and systemic therapy, nearly 80% will have a metastatic relapse [2]. In recent years, there have been significant advances in understanding the biology of pancreatic cancer at the molecular level, including the characterization of the pancreatic cancer genome [3], global expression profiling [4–8], and proteomic analysis [9, 10]. In addition, it has been shown that the dense stroma associated with pancreatic cancer is important in tumor progression and metastasis [11, 12]. Extensive work in this area has demonstrated details of the interaction of supporting cells and cancer cells and of the role of the stroma in creating a barrier to chemotherapy and immunotherapy [13, 14]. This work has provided insight into how more efficacious treatments might be developed in the future. For example, the molecular analysis supports the clinical observation that pancreatic cancer is comprised of different subtypes, each with unique behaviors and responses to therapy. Thus, more effective therapy will need to be developed using a tailored approach that has become known as precision medicine.

One necessary step in the development of a precision approach to pancreatic cancer will require the identification of clinically useful biomarkers. A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of some biological process. An ideal biomarker must demonstrate the ability to accurately function as a surrogate of the biological feature in question. In this

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sense, biomarkers can be derived from pathological specimens or non-pathologic tissues and fluids. The most commonly used biomarkers for the management of cancers are molecular measurements. Examples include tailored therapy for HER2/neu-positive lung cancers [15] or estrogen/progesterone-receptor-positive breast cancers [16].

In the case of pancreatic cancer, very few clinical biomarkers exist. Commonly, carbohydrate antigen 19-9 (CA19-9) is used as an adjunct in diagnosis and as marker of disease course, but has numerous limitations, which are described below. Other markers, such as SMAD4 status or GATA6 upregulation, currently require a tissue biopsy acquired through invasive means. Recently, the concept of blood-based liquid biopsy has been tested and been found useful in assessing cancer biomarkers [17]. Circulating tumor DNA (ctDNA), microsomes, and CTCs, among other factors, can all be identified in a blood sample. In particular, ctDNA and CTCs have shown promise in the search for biomarkers for pancreatic cancer.

The majority of published reports on liquid biopsy for pancreatic cancer have focused on ctDNA [18–20]. However, recent work on CTCs has demonstrated their utility as a possible biomarker, and for some applications, CTCs have advantages over ctDNA [21–24].

First described in the peripheral circulation of a woman with metastatic breast cancer in 1869 by Australian physician Thomas Ashworth [25], CTCs are rare, with 1 CTC per billion normal blood cells per milliliter of blood [26]. Their movement to and persistence in circulation indicates an ability to both migrate away from and survive after detachment from established tumor deposits and suggests that they are an important step in the metastasis of cancer. However, it has been shown that a very small percentage of CTCs contribute to metastatic lesions [27].

CTCs can express both epithelial and mesenchymal characteristics [28–30] and can exist as single cells or clusters of tumor microemboli, which appear to have increased metastatic potential [31]. Their half-life is extremely short, ranging from estimates of 25–30 minutes for single cells and 6–10 minutes for clusters [31] to 1–2.4 hours on average [32]. Thus, CTCs are an opportunity to view the behavior of a tumor in real time, from a single peripheral blood draw, and could possibly be used to both monitor the entire course of disease and more closely explore the dynamics of cancer biology and metastasis.

The purpose of this chapter is to review the current status of CTCs as a biomarker in pancreatic cancer and to additionally discuss the advantages and disadvantages of CTCs as a liquid biopsy.

Liquid Biopsy

A traditional biopsy has been the workhorse of cancer care in terms of establishing a diagnosis and assessment of biomarkers. Unfortunately, traditional biopsies have several limitations. They are a one-time measurement in an entire treatment course; serial sampling throughout the administration of surgical or medical therapy would

be necessary to provide accurate and timely information about changing tumor biology. However, traditional biopsies are invasive, as they require resection or instrumentation of the tumor, meaning it is neither feasible nor practical to perform serial biopsies to guide treatment in real time.

Most tumors, including pancreatic cancer, consist of multiple cellular clones, and each has the potential for a unique biological behavior [33]. This creates a challenge in the choice and monitoring of therapy, as an effective treatment might be introduced early, with a significant initial response, and then fail to maintain results as resistant clones survive and multiply. Tumor heterogeneity also increases the probability of sampling error with the use of traditional biopsy. The inability to detect aggressive clones, which drive outcome, among all subclones has clinical implications in terms of guiding management in a precision approach.

As such, there has been recent interest in the development of biomarkers from bodily fluids – in particular, blood. A liquid biopsy can overcome the limitations of traditional biopsy, as it provides the opportunity to gain access to biomarkers through a minimally invasive method such as a blood draw, with little discomfort and virtually no risks. This advantage goes beyond patient comfort and safety, as a liquid biopsy is amenable to real-time analysis with multiple samples over time to monitor tumor progression and response to therapy. Finally, a liquid biopsy potentially represents the biomarkers of all clones of the primary tumor, metastatic deposits, and subclinical disease. The utility of liquid biopsies has been reported extensively in literature [19, 20, 34].

Currently, no blood-borne biomarker exists for pancreatic cancer that can be used to guide therapy or develop a true liquid biopsy. The most extensively used blood test for pancreatic cancer is CA19-9, which has been shown to be helpful in establishing a diagnosis and in determining recurrence or progression of disease following therapeutic interventions. Beyond these features, CA19-9 is limited in its capacity as a biomarker. Approximately 10% of Caucasians and 22% of African Americans are Lewis antigen negative, rendering this test useless in this population [35–37]. Moreover, CA19-9 is not specific for pancreatic cancer and can be elevated in other cancers as well as in benign conditions such as biliary obstruction, a common concomitant feature in pancreatic cancer [36].

The ideal liquid biopsy for the detection of pancreatic cancer biomarkers would be obtained through a blood draw, represent known intra- and inter-tumor heterogeneity, and give real-time information about the disease course and response to therapy. The ability to perform liquid biopsies for the management of cancer is based on the principle that either cells or molecular markers unique to the tumor are found in the plasma. These include intact cells, free DNA, RNA, and proteins.

The best studied forms of liquid biopsy are ctDNA and CTCs. One method is not superior to the other, and both have shown promise as clinically useful biomarkers in the treatment of pancreatic cancer. The information provided by ctDNA and CTCs is complementary; the isolation and analysis of both provides both the opportunity for a more accurate and extensive understanding of both tumor biology in general, possibly leading to novel therapeutic options, and the ability to predict outcomes in individual patients [34, 38].

While ctDNA, which consists of short DNA fragments released from dying or apoptotic primary tumor or metastatic lesions into blood, is a representation of the genome of all clones of the primary tumor and metastatic sites, CTCs, cells shed from all tumor deposits into circulation, provide not only DNA but also RNA and proteins for analysis. Unlike ctDNA, CTCs have the ability to represent each unique clone present within the tumor and are, as such, not an "average" of the entire disease burden. These cells are the probable source of metastatic lesions; hence, they provide the potential for direct analysis of tumor biology. Moreover, as a manifestation of disease relapse, their analysis may provide a real-time assessment of treatment failure [39–41]. Compared to ctDNA, however, CTCs are less prevalent in plasma, rendering them less sensitive as both a screening marker and for tracking the evolution of disease.

General Methods of CTC Detection

To comprehend the role of CTCs as a biomarker and to interpret literature on the subject, it is important to have a basic understanding of the methods of CTC isolation. Large discrepancies are noted in the data on this subject; much of the variability stems from the abundance of methods used to isolate and identify CTCs. Thus, enumeration and characterization of CTCs taken from the same patient at the same time point can differ depending on the method of isolation utilized.

A number of isolation techniques exist for CTC enrichment, including affinity-based methods relying on antibody-antigen interactions; size-based systems taking advantage of the differences in size between CTCs and other cells in the circulation, such as epithelial tumor cells; negative and positive selection-based approaches, such as flow cytometry; and electric-field-based systems, which separate CTCs by their dielectric properties. Additionally, microfluidic devices work to separate CTCs using laminar flow and allow for detection of multiple properties, such as cell size, deformability, and affinity [42–47].

As CTCs are rare and heterogeneous, isolation can be a challenge. More invasive experimental approaches, such as leukapheresis [48], may heighten the probability of capturing greater numbers of CTCs, but these methods are not ideally suited for clinical use. The type of collection tube, storage and transport conditions, time to analysis, and processing techniques can also impact CTC capture and are especially important when mRNA transcript identification is required or when attempts are being made to culture these cells [49].

Isolation of CTCs presents the additional limitation of possibly excluding cells with certain phenotypes, as current systems designed for this purpose all exploit a specific property of these cells (e.g., surface markers or size). For example, the Veridex CellSearch system (Janssen Diagnostics, Raritan, NJ), approved by the Food and Drug Administration (FDA) in 2004 for CTC detection in breast, prostate, and colorectal tumors, relies on surface EpCAM expression to immunomagnetically capture CTCs, which are further identified as CD45 negative and cytokeratin

(CK) 8-, 18-, or 19-positive cells [50]. However, this system will have limited success in isolating cells with low EpCAM expression. Contrastingly, size-based systems (e.g., ISET, or isolation by size of epithelial tumor cells – Rarecells, Paris, France) [43], while capable of greater sensitivity than platforms such as CellSearch [51, 52], will fail to detect cells smaller than the determined cutoff.

Once a pool of CTCs has been isolated, not only the cell itself but also its DNA, RNA, and proteins are available for analysis for relevant mutations and molecules that might become targets of therapeutic agents. Genomic analysis follows the same principles as for ctDNA with the added need for the extraction of genetic material. RNA and protein characterization allow for a more functional profiling of tumor cells.

CTCs as a Biomarker in Pancreatic Cancer

The prospects for the development of CTC-based biomarkers are immense, and this field is currently in its initial stages. Possibilities include simple enumeration, detailing of subclasses, mutational profiling, and expression profiling of these cells, to name a few. It should be noted that similar work is being done in other cancers – a worse prognosis has been linked to the presence of CTCs in breast cancer [21, 53–58], small cell lung cancer [59, 60], non-small cell lung cancer (NSCLC) [61, 62], cholangiocarcinoma [63], colorectal cancer [64–68], melanoma [69], and prostate cancer [70, 71]. Though CTC research in other cancer types is more well established, a growing body of evidence has demonstrated the predictive value of CTCs in pancreatic cancer. For example, de Albuquerque et al. reported a shorter progression-free survival in patients with CTCs in peripheral blood than in those without CTCs [41]. Similarly, Zhang et al. demonstrated a correlation between CTC positivity and both the development of metastases and worse survival in a cohort that was followed for 18 months [72].

Two meta-analysis reports, each comprising more than 600 patients with pancreatic cancer, have demonstrated a clear correlation between CTC positivity and worse outcomes. One of these studies drew associations between CTC positivity and poorer overall survival (HR = 1.64, 95% CI 1.39–1.94, $p < 0.00001$) and progression-free survival/recurrence-free survival (PFS/RFS) (HR = 2.36, 95% CI 1.41–3.96, $p < 0.00001$), concluding that CTCs can be predictive of pancreatic cancer disease course. CTCs predicted unfavorable outcomes at all time points throughout treatment (before, during, and posttreatment); CTCs were most predictive at the posttreatment time point (PFS/RFS HR = 8.36, 95% CI 3.22–21.67, $p < 0.0001$) [73]. Similarly, a separate meta-analysis, including 623 patients, concluded that CTC-positive patients have worse PFS (HR = 1.89, 95% CI 1.25–4.00, $p < 0.001$) and OS (HR = 1.23, 95% CI 0.88–2.08, $p < 0.001$) [74].

Circulating tumor cells within a given patient exhibit phenotypic heterogeneity, and not all types correlate with outcome. In a series of 50 patients with localized pancreatic cancer who underwent resection, Poruk et al. identified CTCs in 78% of

patients using ISET, followed by negative exclusion of leukocytes using immunofluorescence. In this study, two subpopulations of CTCs were identified – those expressing cytokeratin alone (epithelial-type) and those expressing both cytokeratin and vimentin (mesenchymal-type). On multivariate analysis with typical predictive pathological features and CTC subtypes, there was no correlation between total CTCs or epithelial-type CTCs with recurrence. Interestingly, there was a strong correlation between the mesenchymal-type CTCs and recurrence (HR 2.78 95% CI 1.3–5.9; $p = 0.01$) [29].

These results were further investigated by our group in a longitudinal study called the CLUSTER trial (NCT2974764), where 200 patients undergoing surgical resection of pancreatic cancer were enrolled and CTC concentrations in the patient peripheral blood were measured at fixed intervals, starting prior to surgical resection, at 4 and 6 postoperative days, and every 2–3 months after this time point. In the initial report on the subset of 136 patients who achieved a 12-month median follow-up, CTCs were isolated based on size (>8 microns) and then stratified into epithelial or epithelial-mesenchymal types (Fig. 9.1). Circulating tumor cells were identified in the blood of 131 (96%) patients. The 58% of patients who had received no chemotherapy prior to surgery had significantly higher CTC numbers before resection compared to patients who were post-neoadjuvant therapy (42%). There was a statistically significant decrease in the number of CTCs counted in both the treated and untreated patient populations after surgery; those with early recurrence, defined as recurrence within 1 year of surgery, had significantly higher pre- and postoperative CTC counts and a higher proportion of mixed epithelial-mesenchymal phenotype CTCs. These findings appear to indicate that cells with this epithelial-mesenchymal or transitional phenotype have a more aggressive biology, demonstrating the heterogeneity of CTCs [30]. The epithelial-mesenchymal transition (EMT) is defined as a

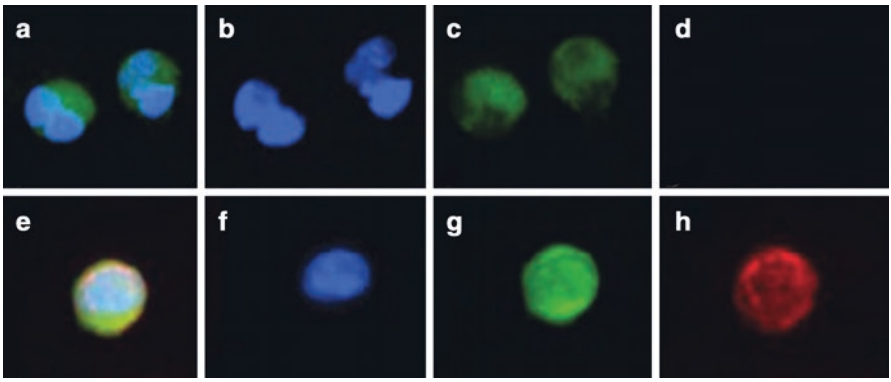

Fig. 9.1 Circulating tumor cells from pancreatic cancer patients. Immunofluorescence microscopy (at 20× magnification) here demonstrating epithelial-like (**a**–**d**) and epithelial-mesenchymal, or transitional (**e**–**h**) circulating tumor cells in patients with pancreatic ductal adenocarcinoma. (**a**) Pan-cytokeratin-positive, vimentin-negative CTC (merged), (**b**) DAPI (blue), (**c**) pan-cytokeratin (green), (**d**) absence of vimentin (red); (**e**) pan-cytokeratin-positive and vimentin-positive CTC (merge), (**f**) DAPI (blue), (**g**) pan-cytokeratin (green), and (**h**) vimentin (red)

reversible phenotypic change where a cancer cell with epithelial characteristics becomes more mesenchymal and invasive in phenotype, distinguished by a down-regulation of E-cadherin, increased expression of mesenchymal markers such as N-cadherin and vimentin, and a loss of its ability to adhere to adjacent cells [75–79]. Prior work in pancreatic cancer has similarly linked EMT to disease dissemination and poorer prognosis [78, 80], and previous studies in other cancer types, such as breast, prostate, and lung carcinoma, have also connected EMT with metastatic disease [28, 81–83].

It is presumed that CTCs are directly responsible for mediating the dissemination of cancer. In order to accomplish this end, these cells must be capable of long periods of quiescence, self-renewal, and differentiation to form a tumor similar to the parent tumor. These are all features of cancer stem cells (Fig. 9.2) [84], also called tumor-initiating cells. Tumor-initiating cells are thought to constitute a small percentage of all cells within cancer (<0.01), but are necessary for driving growth [85]. In animal studies, cancer stem cells are able to establish tumors with as few as 100 cells; to compare, millions of cells from bulk tumor are required to produce the same results. A cancer stem cell from pancreatic cancer expresses CD133, CD44, and aldehyde dehydrogenase (ALDH) [86].

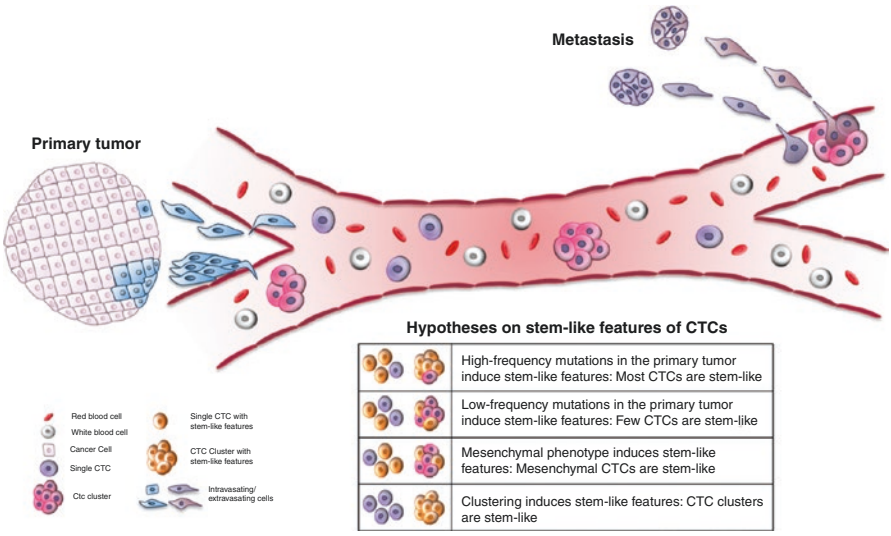

Fig. 9.2 Steps of cancer metastasis. This figure demonstrates what are proposed to be the steps required for the generation of cancer stem cells (tumor-initiating cells) and metastasis. A small percentage of cancer cells within the primary tumor undergo a phenotypic change to become more mesenchymal-like. This change is called an epithelial-mesenchymal transition (EMT) and is associated with cells developing the ability to grow in the absence of contact with the basement membrane, migrate into the circulatory system, and survive the harsh environment of circulation. These cells, now called CTCs, leave circulation through a poorly understood process and take up residence in the stroma of distant organs. A small subset acquire stemlike features; different possible mechanisms of this change are enumerated in the figure. (From Gkountela and Aceto [84], with permission)

Based on the finding of the importance of epithelial-mesenchymal-type CTCs and their correlation with outcome, the hypothesis that a subclass of CTCs would have a stem-cell phenotype was tested by our group. In a cohort of patients undergoing surgical resection, we reported the presence of a CK+/ALDH+ phenotype in 77% of all patients with CTCs at the time of resection. This phenotype predicted worse overall and disease-free survival (HR 3.4, 95% CI 1.2–9.8; $p = 0.03$). In further stratification of this cohort, patients with CTCs that were "triple-positive" for CK/CD133/CD44 had a much higher risk of recurrence compared to those with CK+/CD133+/CD44- cells (6.45, HR 6.45; 95% CI 2.1–19.7).

These results support the idea that at least a subset of CTCs have tumor-initiating cell properties, but there is currently no direct evidence that CTCs from pancreatic cancer are able to form metastases. To show that this is possible, it will be necessary to establish that these cells can be cultured. Attempts to culture CTCs from pancreatic cancer have not yet been successful, but CTC cultures have been reported for some other tumor types, such as colorectal and breast cancers [87–89], and co-culture of CTCs from early stage lung cancer patients using cancer-associated fibroblasts as a template on the CTC-Chip platform has also been reported [46].

Future Potential of CTCs in Pancreatic Cancer

The current literature demonstrates a role for CTCs as a biomarker to predict patient outcomes. However, much work still needs to be done, and the full potential of research in the field of CTCs in pancreatic cancer has not yet been reached. A wealth of information exists in the detailed evaluation of phenotype, expression profiling, and genetic data. Genome analysis of viable and intact CTCs indirectly reflects the tumor of origin. Studies show that sequencing CTC genomes is possible, and high concordance with a clonal relation between CTCs and the corresponding tumor has been found [22, 90].

In 2013, a group from Graz, Austria, was the first to outline a complete genomic profile of CTCs from patients with colorectal cancer through the implementation of array CGH and next-generation sequencing; they compared their findings with a broad panel of 68 known colorectal cancer-related genes and found matching mutations of cancer driver genes (KRAS, APC, PIK3CA) in the primary tumor, in metastatic deposits, and in the corresponding CTCs of the same patients [91]. Other studies confirmed these findings: exome sequencing on patients with lung cancer found that CTCs and tumor metastases had the same mutations [92], and the same held true for prostate cancer [90]. Ni et al. studied the copy number variations (CNVs) of CTCs in patients with lung adenocarcinoma and SCLC and concluded both that CNVs are specific to the type of cancer and that this pattern did not vary with therapy. However, it was also observed that chemotherapy did result in insertions/deletions and single nucleotide variations and that CTCs harbored tumor-related genes, including ones linked to resistance [92]. Therapy can halt cancer growth; nonetheless, it alters the clonal distribution of cancer and confers a selective

advantage to certain cells, coupling the progression of disease with chemoresistance [93].

Similar work is now being reported in the field of pancreatic cancer. For example, RNA expression analysis of pancreatic cancer CTCs in a mouse model identified alterations in the expression of the gene Wnt2, which has been implicated in increasing the metastatic tendency of this cancer [23]. Court et al. analyzed CTCs for KRAS, an oncogene known to be involved at early stages in 95% of pancreatic cancers, and they were able to detect the mutation in 92% of the samples. However, due to difficulties in sequencing and accidental allele dropout during amplification, they were not able to identify KRAS in all the cells and concluded that at least ten CTCs were needed to consistently determine KRAS status due to decreased sensitivity below this threshold number [94]. Another study aimed to examine KRAS in CTCs and interestingly found that patients with detectable KRAS mutations in CTCs had a better survival compared to those with wild-type KRAS in CTCs (19.4 vs. 7.4 months, $p = 0.015$) [95].

The ability to directly evaluate tumor cell phenotype with molecular profiling at diagnosis will be an important and necessary feature for a biomarker for pancreatic cancer to possess. Circulating tumor cells can be assessed at the time of diagnosis and throughout the course of treatment. Since the half-life of CTCs is estimated to be on the order of minutes and as they can be obtained with a simple blood draw, they may prove useful to track subclinical responses to therapy. In this regard, a drop in CTCs has been reported in as few as 4 days following resection [30] and in response to neoadjuvant therapy. In addition, it is possible that the development of chemoresistance could be measured in real time and that patients could be spared months of therapy that will later be found to be ineffective by clinical assessment.

However, the significance of CTCs extends beyond their use as a simple biomarker in that a better understanding of their disease biology may directly improve therapy. Unlike ctDNA, CTCs are a part of the disease process and can essentially be considered as a liquid phase of the tumor. In fact, since the majority of patients with pancreatic cancer die from metastatic disease, CTCs may represent the most clinically important part of the tumor. In patients who undergo surgical resection, the majority of recurrence results from metastases, which are presumed to originate from micrometastatic disease, also called disseminated tumor cells, seeded by CTCs. A better understanding of these cells may help identify unique vulnerabilities that contrast with those of the primary tumor and could result in targeted therapies.

Summary

In the application of precision medicine to pancreatic cancer, novel biomarkers will be necessary to guide therapy. Biomarkers obtained from a traditional biopsy of the primary tumor will be limited in terms of their ability to deliver real-time feedback and to represent and predict tumor behavior as a result of tumor heterogeneity.

Liquid biopsy has the ability to overcome many of these limitations. The use of CTCs as a biomarker in pancreatic cancer has shown initial promise. However, the science regarding CTCs is immature, and to better understand the potential role of CTCs, it is essential to perform further molecular investigations with broader and longer-term studies. More sensitive, advanced, and automated techniques are required to analyze cells from a genetic and molecular perspective and to retrieve a greater number of viable CTCs such that culturing these cells from pancreatic cancer becomes a possibility.

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Part IV
Personalized Treatment Approaches

Chapter 10
Personalized Models of Human PDAC

Hanna Heikenwälder and Susanne Roth

Most patients with PDAC are diagnosed with advanced, unresectable disease, and even highly selected patients with initially limited disease who underwent potential curative resection finally succumb from recurrent disease, while long-term survival remains rare. Although significant progress has been achieved in PDAC patient care, such as neoadjuvant treatment strategies, or more effective combined adjuvant and palliative chemotherapeutic regimens [1, 2], the overall beneficial effect on prognosis has been marginal in unselected patient populations. Currently, chemotherapeutic regimens in pancreatic cancer are still mostly limited by a "one-size-fits-all" approach, meaning that therapeutic decisions are mainly based on the clinical tumour stage and ignoring the patient's individual cancer biology. Recently, high-throughput sequencing technologies have revealed a complex mutational landscape in pancreatic cancer with multiple mutated genes at low prevalence and significant intertumoural heterogeneity [3–8]. Due to the diverse genetic landscape, tumour biology and thus responses to antitumour therapies vary substantially. Although several signature-based mutational and transcriptional subtypes have been proposed in PDAC, so far no reliable biomarker for predicting the effectiveness of antitumour therapies is currently available [2]. Many antitumour therapies are highly toxic and associated with serious side effects. Therefore, it is of central importance to identify those individuals that would benefit from specific antitumour therapies, matching the right treatment to the right patient. Such a personalized treatment strategy could improve prognosis for patients with this devastating disease and also reduce therapy-associated toxicity. Yet, response prediction to antitumour drugs remains a major challenge in cancer treatment. Prediction based solely on cancer genome sequencing is limited, and recent evidence indicates that intratumoural heterogeneity and the tumour

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microenvironment can restrict biomarker-guided strategies for therapy selection. This limitation could be addressed by direct functional response testing of live patient tumour tissues exposed to potential therapies. Several functional assays assessing antitumour activity of drugs have been developed, such as stable tumour cell lines, organoids, or xenograft models from individual patients [9], which possess unique drug sensitivity profiles that could not be predicted using genetic analyses [10]. Personalized models of PDAC should recapitulate the genetic complexity and heterogeneity of the disease and prevent the process of adaption to in vitro growth conditions that leads to significant changes in the biology of cancer cells. Those models might help to choose the right therapy in a clinically relevant time frame, enabling more effective individualized treatment options to improve outcomes for patients with pancreatic cancer and prevent the unnecessary use of chemotherapeutics to which patients are resistant, thereby reducing toxicity. In this chapter, we provide a brief overview of the most promising personalized models of human PDAC, including patient-derived xenograft models, cell lines, organoids, tumour tissue slice cultures and circulating tumour cells, as well as potential future applications (Fig. 10.1).

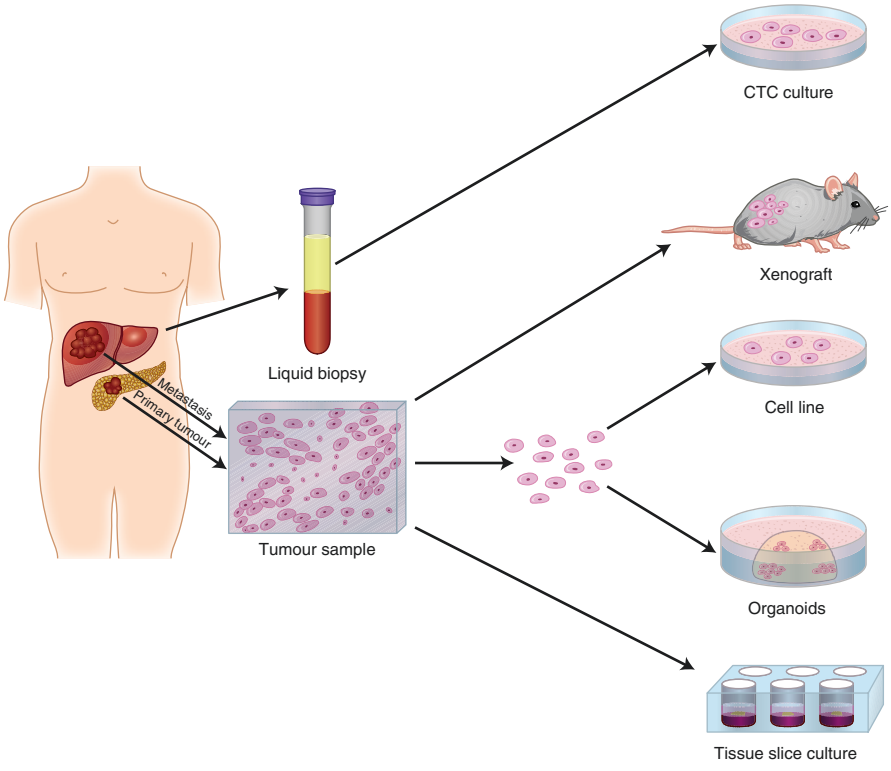

Fig. 10.1 Personalized models of human PDAC. Tumour specimen and liquid biopsies from PDAC patients can be used to generate individualized tumour models, including xenografts, patient-derived cell lines, 3D organoids, tissue slice cultures and cultures of circulating tumour cells, respectively. In-depth molecular and functional characterizations of these models help to select most effective therapies in each individual patient

Xenograft Models

Xenograft models have been used for decades in preclinical research as a valuable tool to study tumour biology and for drug screening. Xenografts can be generated from small pieces of primary human tumours and metastases collected by surgery or even from biopsy samples. These small tissue pieces are then transplanted either subcutaneously or orthotopically into immunodeficient mouse strains such as athymic nude or NOD-SCID mice. Orthotopic transplantation refers to transplantation of the original tumour tissue into the corresponding anatomical location in the mouse, e.g., primary human PDAC tissue into the murine pancreas. Although the orthotopic approach would most accurately mimic the natural tumour microenvironment, subcutaneous transplantation into the dorsal flank of immunodeficient mice is the standard procedure. This is mainly due to practical reasons, as orthotopic transplantation does not only require advanced surgical skills and is more time-consuming but also makes it much more difficult to monitor the engraftment success and tumour growth. Patient-derived xenograft models are a suitable tool for personalized treatment approaches in PDAC, as they allow studying tumour cells in their highly heterogeneous environment, composed of a dense extracellular matrix and other cell types that typically reside within these tumours. However, studies have shown that the non-malignant cell types of patient-derived xenografts are substituted over time by murine cells [11]. The cells that infiltrate the xenograft have been shown to be overall the murine counterparts of the original stroma and even produce comparable extracellular matrix components [12–14]. Nonetheless, the substitution of human by murine stroma might interfere with the original interaction of tumour cells with their microenvironment and impose a selective pressure on cancer cells towards an adaption to the new environment. In addition, xenograft models require the use of immunocompromised mice, which prevents the analysis of tumour interactions with the immune system that plays a pivotal role in PDAC development. Human PDAC displays an immune cell signature that is commonly highly immunosuppressive and is thought to be a major contributor to its poor prognosis [1]. Furthermore, the immunocompromised background of mouse strains employed for xenograft generation renders it difficult to test immunotherapies such as checkpoint inhibitors. A potential solution to this problem might be the generation of humanized mice that are irradiated prior to xenograft transplantation and reconstituted with bone marrow containing haematopoietic stem cells from the individual human xenograft donor [15, 16].

Despite these apparent drawbacks, patient-derived xenograft models show gene-expression profiles that are similar to the original tumour [13], and chemotherapy response rates are comparable between patient-derived xenografts and clinical data [13]. Notwithstanding, patient-derived xenograft models have considerable limitations for the use of personalized treatment approaches such as chemosensitivity testing. Especially the low engraftment rates and time required for growth in the recipient mouse remain an unsolved problem. Some approaches tried to increase engraftment rates by transplanting tissue pieces coated with Matrigel or additional

cell types such as human fibroblasts or mesenchymal stem cells. Interestingly, patient-derived xenograft engraftment rates in PDAC showed to be similarly low for subcutaneous or orthotopic transplantation protocols in nude mice with 61% and 62%, respectively [17, 18]. Generally, metastases were found to have higher engraftment rates than primary tumours [13], and successful engraftment was shown to be associated with worse recurrence-free and overall survival in PDAC [19]. These engraftment rates are definitely too low to allow for reliable individualized drug testing or co-clinical trials analysing the underlying mechanisms of treatment responses. Another major problem is the long engraftment time of 4–8 months before drug testing and analyses can be performed [20]. Especially in PDAC, this time gap between initial surgery and start of adjuvant therapy is unacceptable [2].

Patient-Derived Cell Lines

Patient-derived tumour cell lines are generated from human PDAC specimen by tissue dissociation producing single cell suspensions. Once isolated from the fresh tumour sample, the cells first need to adapt to growth in serum containing media in tissue culture dishes, which is the main critical step in the generation of tumour cell lines. Patient-derived cell lines are usually established only from more aggressive tumours and hence are not representative of the full clinical spectrum of PDAC in humans [13]. For PDAC, the efficiency to generate cell lines from a resected primary tumour is even lower than the efficiency of generating 3D cultures such as organoids [21]. In most cases this excludes the generation of cell lines from patients that are diagnosed with early disease. Thus, at the moment being patient-derived cell lines seem to be a rather insufficient strategy for personalized treatment applications [13]. Once patient-derived tumour cells have successfully adapted to their new in vitro environment, they are easy to culture, passage, cryopreserve and manipulate chemically and genetically [11]. Yet, this adaption also represents a major disadvantage, as it induces fundamental changes in cell physiology such as altered gene expression. Newly developed pancreatic cancer cell lines seem to harbour strong genetic conservation with the primary tumour, but this conservation is diminishing with increasing numbers of passages [22]. Thus, low passage cell lines could be used to dissect therapeutic vulnerabilities based on genetic features of individual PDAC samples. Furthermore, the development of various cell lines derived from distinct subclones of the same primary tumour specimen would allow to study tumour heterogeneity. Still, those monolayer forming cell lines fail to recapitulate many key features of the primary tumour, such as 3D organization, interactions with fibroblasts, immune cells and the ECM [11]. Despite these apparent disadvantages, patient-derived cell lines still hold some benefits over other personalized models of human PDAC. The development of patient-derived cell lines is relatively cost-effective, easy to handle and feasible within a time frame that allows the employment of the obtained information for treatment choices, however with the exclusion of those patients, whose tumour cells do not adapt to growth under culture conditions.

Organoids

The limitations of monolayer cell cultures have inspired the development of more physiological organoid cultures, in which cells grow in 3D structures inside or on top of matrices that compensate for the primary ECM. 3D culture methods prevent cells from attaching to the cell culture dish, enable PDAC cells to develop polarity and organize into ductlike structures resembling the original PDAC architecture. Yet, those organoids have been missing stroma components. Recently, more complex organotypic cultures of tumour, stromal and immune components of the original tumour microenvironment have been successfully generated [23]. Tsai et al. co-cultured primary PDAC cells with cancer-associated fibroblasts from the same tissue and lymphocytes from peripheral blood, which infiltrated the 3D in vitro models [23]. Besides collagen type I and IV, Matrigel is the most commonly used matrix for PDAC organoid growth [11]. While collagen type IV is a major component of the basement membrane of the normal pancreatic epithelium, collagen type I is excessively produced by the PDAC microenvironment forming a dense desmoplastic stroma. Matrigel is a commercially available basement membrane extract that is purified from murine Engelbreth-Holm-Swarm (EHS) sarcomas [11, 24] and contains a complex composition of structural proteins (e.g. collagen and laminin) and various growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) [24]. This highly physiologic composition of Matrigel allows patient-derived PDAC cells to form organoids that resemble primary human PDAC much stronger than monolayer cultures [11].

Organoids enable the propagation of large amounts of tumour tissue, providing sufficient material for in-depth genetic and molecular characterisation, as well as drug screening [11]. The efficiency to generate organoids from resected primary PDAC specimen (around 75%) is mostly higher than for cell lines or xenografts [21, 25]. In addition, organoids can be generated from relatively few, or even single, tumour cells [11], which enables the utilization of cancer cells that are obtained by fine needle biopsies and thus allows the generation of personalized tumour models also from PDAC patients that are not eligible for surgical resection [26]. Furthermore, organoids derived from human PDAC can show several distinct morphologies within the same culture [27] and might allow comprehensive modelling of the full spectrum of the disease [3]. Multiple distinct organoid cultures could be generated from distinct geographical regions of the same tumour sample to model intratumoural heterogeneity observed in human PDAC [28].

Patient-derived organoids have already been established as drug testing pipelines for therapeutic profiling in PDAC within a clinically meaningful time frame of less than 6 weeks [25], and the results of a small retrospective analysis confirmed that drug sensitivities observed in patient-derived organoids resemble clinical responses and may thus be used to inform treatment selection [25].

Tumour Slice Cultures

Tumour slice cultures appear to be so far the most physiological approach to model and study individual human PDAC. In contrast to xenografts or cell lines, they exclude species differences and cellular alterations due to adaption to in vitro growth conditions. For the generation of tumour slice cultures, fresh patient-derived tumour samples are cut directly after surgical resection into very thin tissue slices and cultured in the presence of nutrient and growth factor containing media. Membrane filter inserts coated with collagen can be used in order to improve nutrient exchange between slice cultures and culture media and support oxygenation of the tumour slices [29]. The emergence of microtomes with vibrating blades (vibratomes) has enabled the cutting of fresh primary PDAC tissue without the usage of embedding media or fixatives. An ideal slice thickness of 200–400 μm allows for sufficient nutrient diffusion while preserving the original morphology, cellular composition and extracellular matrix components of the primary tumour microenvironment. Therefore, tumour slice cultures also contain various tumour cell subclones that account for tumour heterogeneity observed in human PDAC. A major advantage of tumour slice cultures is that once established, they can be used without any restrictions on any available tumour sample in contrast to xenografts or 2D/3D cell lines, which depend on engraftment success and adaption to growth under culture conditions. However, tumour slice cultures can only be developed when sufficient material is available, and this largely excludes any patient who is not eligible for surgical resection. Tumour slice cultures maintain their baseline morphology and show stable amounts of total cell numbers and a high degree of tissue viability for up to 7 days in culture [29, 30]. In addition, tumour intrinsic immune cell populations such as T cells, macrophages and myeloid-derived suppressor cells were present throughout the culture period [29, 30]. A time frame of 5–6 days is short when compared to other personalized models of PDAC, however sufficient to allow drug testing that might help to identify the optimal treatment for individual patients. As tumour material is limited, tissue slice cultures are not suitable for large-scale drug screens. Noteworthy, also tumour slice cultures are generally produced from only one single region of a human PDAC specimen. In order to address tumour heterogeneity ideally, multiple distinct regions of a single PDAC specimen should be used for the establishment of drug response platforms in precision medicine practices. Further improvements in slice techniques might allow developing tumour slice cultures even from biopsies. Data obtained from these cultures could be used to inform neoadjuvant or palliative treatment choices.

Circulating Tumour Cells

Recently the isolation and culture of circulating tumour cells (CTCs) from peripheral blood of cancer patients has become feasible. The great advantage of this approach in comparison to other personalized models of PDAC lies clearly within

its minimal invasiveness [31]. CTCs can be analysed immediately after isolation or stored by "vitrification" – a method of rapid and "ice-free" cryopreservation [32]. The major drawback of CTCs is their paucity, with concentrations as low as 1 CTC in 10^9 blood cells [32]. CTCs have been successfully purified using label-free microfluidic or filter technologies that allow size-based isolation of viable CTCs with depletion rates of white blood cells of up to 99.99% in whole blood [33–35]. These techniques allow fast processing of large patient blood volumes in a cost-effective manner [35]. In addition, antibody-based enrichment methods are applicable such as positive immunoselection for CTC surface markers (e.g. for epithelial cell adhesion molecule; EpCAM) or negative selections depleting leucocytes, usually via anti-CD45 antibodies [36]. The low numbers that are obtained from patient blood make CTCs difficult to culture and expand in vitro. Several studies have recently established culture conditions that enable functional analyses of CTCs in several malignant diseases [31]. While CTCs have been widely used as biomarkers and for molecular characterization of human individual PDAC, only a few studies exist, which have successfully cultured and expanded human CTCs from PDAC ex vivo [37, 38]. Although CTCs cannot be detected in the peripheral blood of all PDAC patients, CTCs have been captured from blood samples of patients with early, advanced or metastatic disease with high efficiency [34, 39]. Once established, the ex vivo culture and expansion of CTCs might be an easy and noninvasive option for testing the sensitivity to drugs in PDAC patients at all disease stages and during the course of therapy over time.

Future Personalized Models of Human PDAC

Since human PDAC shows high genetic heterogeneity and multifaceted interactions with a complex and immunosuppressive tumour microenvironment, future personalized models of human PDAC should preserve most of its original features while supporting survival or even expansion of the tumour tissue ex vivo. New culture methods such as organoids and latest organ-on-a-chip technologies appear to be promising candidates to comprehensively model human PDAC in vitro, but have until now only been partly successful. Even organ-on-a-chip systems, which try to recapitulate the 3D structure of organs and integrate dynamic properties of live tissue, still remain artificial and fail to recapitulate the whole in vivo composition of tissues [40]. Thus, human organ cultures such as patient-derived tumour slice cultures described above might offer a simple and accurate approach to model individual PDACs with practicability on every patient from whom sufficient tissue can be obtained. However, organ cultures are still limited as they only model local disease processes. No technique is currently capable of comprehensively imitating antitumour immune responses in vitro. Also, multi-organ interactions, off-target or systemic side effects of new drugs cannot be infallibly predicted by any model system.

Worldwide, extensive research focuses on the establishment of personalized models of PDAC as a crucial part of precision medicine approaches. However,

besides technical challenges that still need to be overcome, there are also numerous practical and organizational problems to be solved in the future. Most importantly, data obtained from personalized models of PDAC would need to be collected and shared between institutions and countries worldwide in order to surmount small cohort sizes for rare genetic alterations. Ideally, data would be centralized in specialized national and international centres with unified definitions for patient and data collection [1].

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Chapter 11
Therapeutic Targeting of Stromal Components

Albrecht Neesse

The tumor bulk in pancreatic ductal adenocarcinoma (PDAC) is composed of large amounts of tumor stroma [1] (Fig. 11.1). The tumor stroma or the tumor microenvironment (TME) is a term that describes a complex and highly heterogeneous composition of various nonneoplastic cells and acellular matrix components that surround and embed neoplastic cells, thus shaping a biophysically hard and stiff, yet biochemically highly dynamic matrix scaffold [2–4]. Cancer-associated fibroblasts (CAFs) that originate from various subgroups of myofibroblastic cell types such as pancreatic stellate cells (PSCs) [5–7], resident fibroblasts, mesenchymal stem cells (MSCs), and endothelial cells constitute an important cellular part of the TME [8–10]. CAFs are not a homogeneous cell population itself, and recent reports are only starting to unravel the complexities of several subgroups of CAFs [11], i.e., tumor-promoting CAFs (inflammatory CAFs, iCAFs), tumor-restraining CAFs (myofibroblastic CAFs, myCAFs) [12], or antigen-presenting CAFs (apCAFs) that express MHC class II and CD74 [13] (Fig. 11.2). Besides CAFs, immune cells such as cytotoxic T cells, mature dendritic cells (DCs), macrophages (M1 + M2 subtype), natural killer cells (NKs), myeloid-derived suppressor cells (MDSCs), and T-regulatory cells (Tregs) abundantly accumulate within the stroma and often create an immunosuppressive environment that impedes clearance of neoplastic cells [8]. However, detailed compositions of immune cells and the various emerging immune therapies will be discussed elsewhere in this book. Besides immune cells and CAFs, endothelial cells and neurons can also be found in the TME [14, 15]. Cells from the TME closely interact with epithelial tumor cells either through direct cell-cell interactions and subsequent activation of signalling pathways or via abundantly released growth factors, hormones, and cytokines that generate a highly complicated

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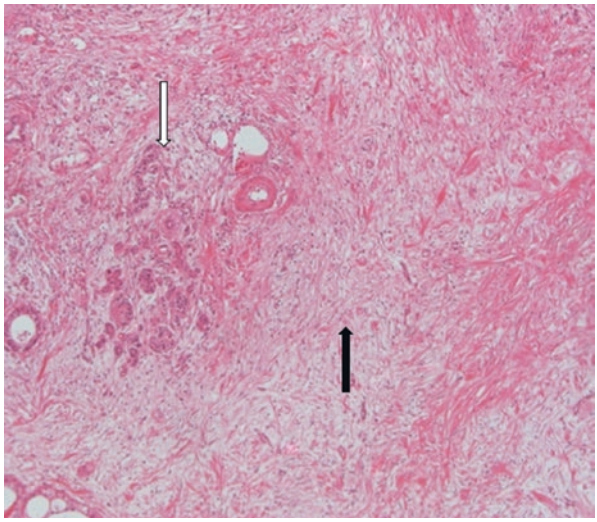

Fig. 11.1 H&E staining of a human pancreatic ductal adenocarcinoma (PDAC) with a pronounced tumor stroma (black arrow) and small nests of neoplastic cells (white arrow)

communication network [16, 17]. Apart from the cellular components, the acellular matrix components make up a large part of the bulk tumor volume. Among others, collagen, hyaluronic acid (HA), fibronectin, and matricellular proteins such as secreted protein acidic and rich in cysteine (SPARC), periostin, and tenascin C can be found in the TME [18, 19].

Historically, the tumor stroma was considered a fibrotic scar that surrounds and confines neoplastic cells, thus rather preventing than promoting tumor progression and spread of neoplastic cells to distant organs [1]. Several decades ago, however, it became increasingly clear that the TME coevolves with transformed epithelial cells in several carcinomas including PDAC. Since then, numerous studies have provided evidence that the TME is a highly dynamic, heterogeneous, and complex arrangement of cells, growth factors, and matrix components that promote tumor progression, spread, and therapeutic resistance through a complex biochemical and biophysical cross talk with neoplastic cells [3, 20]. From a clinical point of view, therapeutic resistance toward chemotherapies remains a major challenge in the oncological care of PDAC patients, and despite the emergence of novel, intensified chemotherapies such as nab-paclitaxel + gemcitabine [21] or FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin) [22], most patients with advanced or metastasized disease die during the first 12 months after diagnosis [16]. Therefore, the idea that the extensive desmoplastic reaction in PDAC could be causally involved in therapeutic resistance and hence serve as a therapeutic target seems highly appealing for scientists and clinicians likewise. However, more than two decades between “hope and hype” for anti-stromal therapies have passed without the emergence of a single, clinically approved anti-stromal compound [8]. Despite this apparent lack of bench-to-bedside translation, anti-stromal approaches

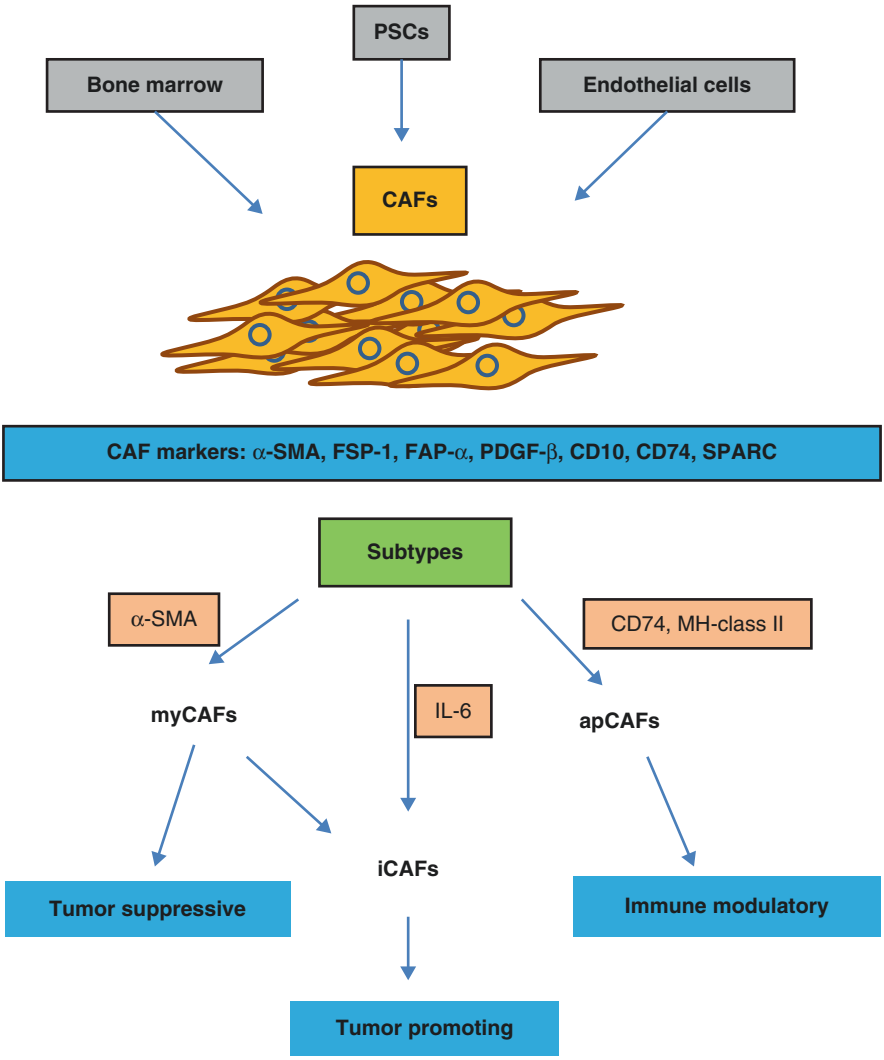

Fig. 11.2 Cancer-associated fibroblast (CAF) evolution, CAF markers, and subtypes in PDAC according to current knowledge. PSC, pancreatic stellate cells; α-SMA, α-smooth muscle actin; FSP-1, fibroblast-specific protein-1; FAP-α, fibroblast activation protein-α; PDGFR-β, platelet-derived growth factor receptor-β; SPARC, secreted protein acidic and rich in cysteine; myCAF, myofibroblastic CAFs; iCAF, inflammatory CAFs; apCAFs, antigen-presenting CAFs

may still constitute powerful and clinically relevant therapeutic options for at least a subset of PDAC patients, and this article will discuss reasons and obstacles that may have led to such apparent failure of preclinical to clinical translation in the past.

Targeting the Tumor Matrix and Stromal Signalling Pathways

The first "hype" for anti-stromal therapies in PDAC was initiated by promising preclinical and early clinical trial results from the broad-spectrum synthetic matrix metalloproteinase (MMP) inhibitor marimastat [23]. MMPs are proteolytic enzymes within the TME predominantly expressed from activated CAFs and involved in the dynamic remodelling and turnover of extracellular matrix (ECM) proteins [24, 25]. In particular, MMP-2 and MMP-9 are highly expressed in PDAC and exert additional pro-migratory and pro-invasive functions [26, 27]. However, the hope of a first anti-stromal therapy was soon squashed by several phase III trials with marimastat as well as selective MMP-2, MMP-3, MMP-9, and MMP-13 (Bay-12-9566) that did not show efficacy in PDAC patients alone or in combination with gemcitabine suggesting a more complex biology of MMPs than initially anticipated [28, 29].

Almost 10 years later, a seminal preclinical study by Olive et al. fuelled great hope and optimism that stromal targeting might be a key strategy to improve response to standard chemotherapies [30]. In this study, the group of David Tuveson pharmacologically inhibited the sonic hedgehog (SHH) pathway in genetically engineered mice that closely recapitulate the stromal composition of human PDAC. SHH is a crucial signalling pathway that centrally controls the cross talk between tumor cells and surrounding stromal cells and promotes tumorigenesis. In particular, tumor cells release SHH ligands that in turn act in a paracrine mode on mesenchymal cells [31, 32]. Using the pharmacological inhibitor IPI-926, the Tuveson group showed pronounced depletion of the tumor stroma that was accompanied by an increase in vessel density and patency. Combination with gemcitabine resulted in higher intra-tumoral levels of gemcitabine, increased rate of apoptotic tumor, cells as well as prolonged survival in tumor-bearing genetically engineered mice (GEMMs) [30]. This hallmark study introduced the tumor stroma as a biophysical and hypovascular barrier for the accumulation of chemotherapy and suggested that stromal depletion strategies would be able to sensitize PDAC to standard chemotherapies. However, despite these promising preclinical data, the clinical trials for SHH inhibitors failed at early stages or had to be suspended prematurely due to decreased survival rates caused by SHH inhibitors [33, 34]. Subsequent in-depth studies of the SHH pathway in various GEMMs revealed that a prolonged inactivation of the pathway resulted in ablation of CAFs, but undifferentiated and more invasive and aggressively growing pancreatic tumors [35, 36]. Very recent data show that *Smo* deletion in fibroblasts leads to increased tumor cell proliferation and proteasomal degradation of the tumor suppressor PTEN with subsequent activation of oncogenic protein kinase B (AKT) in fibroblasts [37]. Interestingly, low stromal PTEN correlated with reduced overall survival in PDAC patients [37]. These studies received great attention in the pancreatic cancer community as they suggested for the first time that stromal cells, in particular CAFs, mediate tumor-restraining rather than tumor-promoting properties. Subsequently, the therapeutic strategy was reconsidered and "stromal reprogramming" rather than "stromal depletion"

appeared to be more appropriate. To this end, a number of exciting preclinical studies were recently published that provide insights into stromal biology and the potential to alter biophysical or biochemical properties of the matrix in order to regulate tumor cell tension and contractility and improve therapeutic response [38, 39]. For instance, actomyosin contractility was downregulated in PSCs by all-trans retinoic acid (ATRA), the active metabolite of vitamin A via the retinoic acid receptor [40]. Alternative stromal targets include Rho-kinase (ROCK) [41], the TGF-β pathway [42], and lysyl oxidase (LOX) [43], which have all been tested in preclinical experiments mostly involving various GEMMs. However, translation from preclinical to clinical findings and back is often difficult and exemplified by the stromal depletion hypothesis that was incorrectly suggested for nab-paclitaxel. Nab-paclitaxel is an albumin-coated, nano-formulated drug that was shown to significantly prolong survival in combination with gemcitabine in stage IV PDAC patients in a large, multinational phase III trial [21]. SPARC is an albumin-binding protein that is overexpressed by peritumoral fibroblasts in PDAC [44]. Early clinical trial data and preclinical data indicated that stromal expression of SPARC may predict efficacy of nab-paclitaxel possibly by specifically increasing intratumoral drug accumulation through binding of SPARC and nab-paclitaxel [45]. Furthermore, the interaction between SPARC and nab-paclitaxel was suggested to deplete tumor stroma, thus breaking down the stromal barrier and increasing the accumulation of other drugs such as gemcitabine [46]. However, neither the stromal ablation theory nor the value of SPARC as biomarker for nab-paclitaxel treatment could be confirmed in controlled clinical trials, appropriate GEMMs using SPARC-knockout alleles, and patient-derived xenografts [47–49]. Therefore, the remarkable efficacy of nab-paclitaxel and gemcitabine in PDAC is likely due to improved tolerability of higher doses due to the albumin formulation and possibly also by decreasing the levels of the gemcitabine inactivating enzyme cytidine deaminase in tumor cells [50].

Although stromal depletion approaches have so far failed to achieve meaningful clinical efficacy and caused scepticism to whether it is the most promising therapeutic strategy, the heterogeneity of the stromal composition in human PDAC might be one reason for the conflicting results in murine models and human PDAC. Even though GEMMs recapitulate the tumor stroma quite closely, they are still distinctly different from human PDAC and most likely not as heterogeneous as previously anticipated. Therefore, PDAC patients might benefit from stromal subtyping approaches that could identify certain stromal targets for pharmacological modifications. To this end, pharmacological depletion of hyaluronic acid (HA) by enzymatic degradation using PEGPH20 in combination with gemcitabine was shown to be successful in GEMMs [51, 52]. Interestingly, the corresponding phase I/II study (NCT01839487) showed robust response rates for those patients that highly express HA in the tumor stroma indicating a potential first step toward personalized anti-stromal therapy [53]. Results from a large, randomized phase III trial (NCT02715804) using PEGPH20 in combination with nab-paclitaxel and gemcitabine in stage IV PDAC patients with high levels of HA have not been published yet but are expected to be negative.

Therapeautic Targeting of CAFs

Besides immune cells, CAFs are the predominant cell type within the activated, highly dynamic TME in PDAC [11]. CAFs have been implicated in therapeutic resistance by releasing abundant growth factors and cytokines that act upon surrounding stromal and tumor cells. CAFs are also directly involved in drug metabolism of gemcitabine. To this end, PSCs and CAFs were shown to metabolize and accumulate gemcitabine metabolites intracellularly, thus scavenging large quantities of active metabolites that are not available for tumor cells anymore [54]. Therefore, CAFs are an extremely promising therapeutic target; however, great attention should be paid to the selection of CAF targets as recent preclinical data indicate that broad depletion of CAFs may cause opposite effects with decreased tumor cell differentiation, increased invasiveness, and aggressiveness [35, 36]. Therefore, emerging knowledge about subtyping of CAFs will assist therapeutic target discovery to selectively inhibit tumor-promoting CAFs (e.g., iCAFs) and spare tumor-restraining CAFs (e.g., myCAFs) (Fig. 11.2). The abundance of poorly defined CAF markers such as α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP-1), fibroblast activating protein-α (FAP-α), platelet-derived growth factor receptor-β (PDGFR-β), CD10, CD74, or SPARC and the potential of CAF plasticity with dynamic states of subtypes pose additional challenges (Fig. 11.2). However, first data are emerging that describe therapeutic targeting of CAF signalling pathways or receptors in experimental models of PDAC. For instance, the vit D receptor (VDR) on PSCs can be activated through VDR ligand calcipotriol and leads to subsequent reduction of PSC activation and fibrosis, thus reprogramming the TME [55]. Similar data regarding the induction of a more quiescent and less motile PSC phenotype were published in vitro and in vivo using ATRA [56]. Ongoing clinical trials in the USA and the UK are currently investigating the safety and efficacy of ATRA and paricalcitol in combination with chemotherapy in PDAC (NCT03307148, NCT03520790). Apart from the vit D receptor, somatostatin receptors (sst1-sst5) are G protein-coupled receptors that are selectively expressed on CAFs and might serve as therapeutic targets in PDAC. To this end, sst1 is overexpressed on CAFs in PDAC and can be pharmacologically targeted by SOM230 (Pasireotide® Novartis) that activates sst1 and subsequently blocks the mTOR/4E-BP1 pathway in CAFs [57, 58]. Targeting of the mTOR/4E-BP1 pathway subsequently led to sensitization to gemcitabine and inhibition of cancer metastasis via Il-6 and other CAF secreted factors [58]. A phase I study (NCT01385956) evaluated Pasireotide in locally advanced and metastatic PDAC (n = 20 patients) and found the compound to be well tolerated [59].

CAF-derived exosomes have recently been discovered to mediate chemoresistance in tumor cells via *Snail*, and therapeutic inhibition of exosome release by GW4869 showed beneficial therapeutic effects in co-culture experiments [60]. Further in vivo investigations and possibly clinical trials in combination with chemotherapy are required to comprehensively evaluate inhibition of exosomes as CAF-targeted therapies.

Nanoparticle formulations have also been attempted to selectively kill CAFs. For instance, carboxymethylcellulose-docetaxel nanoparticle (Cellax™-DTX) improved therapeutic response and selectively accumulated in α-SMA-positive CAFs in xenograft models [61]. However, regarding the various molecular subtypes of CAFs that are currently emerging and the use of xenograft models that poorly recapitulate the TME, doubts remain whether this strategy may be sophisticated enough to exclusively target tumor-promoting CAFs.

Another potentially CAF inactivating drug is Minnelide, a water-soluble prodrug of triptolide, a derivate from the Chinese plant *Tripterygium wilfordii*. Following promising experimental data in GEMMs [62], Minnelide is currently investigated in a clinical trial in PDAC patients (NCT03117920).

CAFs not only govern ECM composition and cross talk with tumor cells but also critically affect the composition and function of immune cells. The interaction between CAFs and immune cells often leads to an immunosuppressive TME, thus offering vantage points for future therapies alone or in combination with modern immune therapeutics such as checkpoint antagonists [63]. To this end, the subpopulation of FAP-α CAFs was discovered as major source of CXCL12 that mediated immunosuppression. Using a CXCL12 receptor chemokine (C-X-C motif) receptor 4 inhibitor (AMD3100), rapid T-cell accumulation and response to T-cell checkpoint inhibitors was reported in GEMMs [64]. Two early clinical dose escalation trials (NCT03277209, NCT02179970) were performed recently with AMD3100 (Plerixafor, Sanofi Oncology) to assess the safety, tolerability, and effects on the immune microenvironment, but results have not been reported so far. Table 11.1 summarizes clinical trials and preclinical evidence discussed above.

Table 11.1 Selection of currently active or recently completed clinical trials targeting various stromal components derived from preclinical evidence

NCT number	Phase	Target	Compound	Co-treatment	Preclinical evidence (Ref.)
NCT03117920	II	CAF inactivation	Minnelide	–	[62]
NCT03307148	Ib	CAF inactivation	ATRA	Gemcitabine + nab-paclitaxel	[40, 56]
NCT03331562	II	Vit D receptor agonist	Paricalcitol	Pembrolizumab	[55]
NCT02715804	III	Enzymatic degradation of hyaluronic acid	PEPGH20	Gemcitabine + nab-paclitaxel	[51, 52]
NCT03277209 NCT02179970	I	Antagonist for CXCR4	Plerixafor	–	[64]
NCT01385956	I	Agonist for somatostatin receptor 1	SOM 230 LAR (Pasireotide)	Gemcitabine	[57, 58]

CAF cancer-associated fibroblasts, *CXCR4* C-X-C motif receptor

Concluding Remarks and Future Directions

This article attempts to summarize important developments and current knowledge in the field of therapeutic targeting of the PDAC tumor stroma with a particular focus on acellular matrix components/signalling pathways and CAFs. Despite numerous successful preclinical trials, anti-stromal therapies have so far failed to play a role in clinical routine due to the lack of approved therapies. There are several reasons for this failure: (i) Tumor stroma constitutes a very heterogeneous mass and greatly differs in function and composition among patients. (ii) A variety of model systems have been used in the past to test therapeutic targets and novel drugs (i.e., xenograft models, patient-derived xenografts, orthotopic tumor transplantation, various GEMMs, and more recently organoids). Each of the model systems recapitulates certain features of the TME, but none is universally valid and predictive, and results are often hard to translate from one model to the other. (iii) Treatment schedules and length of treatment are fundamentally different in mouse trials and can only capture a small proportion of real-life oncological treatment outcome in patients as seen for SHH inhibition. (iv) Preclinical trials have no general regulation of required (double-blinded and randomized) controls and often lack sufficient sample size. (v) The complexity of interaction and interdependency of single stromal targets or cells with multiple signalling pathways has been underestimated.

Molecular subtyping of PDAC aims to address the genetic complexity by using high-throughput sequencing technologies such as whole-genome profiling and transcriptome profiling [65]. This molecular taxonomy might provide fundamental molecular characteristics of tumors that are otherwise microscopically indistinguishable. In analogy of the predominant epithelial subtypes [66, 67], “activated” and “normal” stroma subtypes were recently described and independently prognostic [68, 69]. Further discoveries in this field will aim to establish therapeutic implications and individualized therapeutic strategies according to the predominant molecular subtype. Accordingly, clinical trials will have to tackle these issues by providing powerful translational programs to maximize gain in insight. Furthermore, umbrella and basket trials might be more appropriate to identify effective treatment approaches in small subgroups of PDAC patients rather than the traditional phase I–III algorithm.

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Chapter 12
Epigenetic Targeting

Svenja Pichlmeier and Ivonne Regel

Historically, pancreatic cancer studies have focused on genetic mutations and classified four frequently mutated genes including *KRAS*, *CDKN2A* (p16), *TP53*, and *SMAD4* as "drivers" for pancreatic carcinogenesis. Numerous other "passenger" mutations with a lower prevalence have been identified with next-generation sequencing (NGS) techniques and affect, among others, epigenetic remodeling enzymes that catalyze histone modifications (*KDM6A*, *SETD2*, *MLL2*, *MLL3, ARID1A*, *SMARCA4*) [1, 2]. However, the genetic variants cannot fully explain the different phenotypes of pancreatic cancer, characterized by molecular patterns, therapy resistance, or metastasis formation. Moreover, pancreatic cancer subtypes, defined through gene expression profiles, did not show an association to the identified somatic mutations [1, 3, 4]. In an emerging set of preclinical studies, researchers have investigated epigenetic profiles in pancreatic cancer, which reveal a correlation to pancreatic cancer phenotypes and their characteristics [5, 6]. Consequently, a consideration of the epigenetic status in PDAC tumor tissues could be relevant for prediction and clinical outcome. The reversible nature of epigenetic modifications and a reprogramming of the epigenetic landscape toward a less aggressive tumor phenotype harbor a great potential for pancreatic cancer treatment.

Epigenetics describes structural adaptions in chromatin states that contribute to gene activity without changing the underlying DNA sequence [7]. Particularly, DNA methylation and posttranslational histone modifications, such as methylation or acetylation, determine an open or closed chromatin conformation that is associated with transcriptional active or repressive gene loci (Fig. 12.1). Notably, the epigenetic landscape is highly changed in malignant cells. Probably

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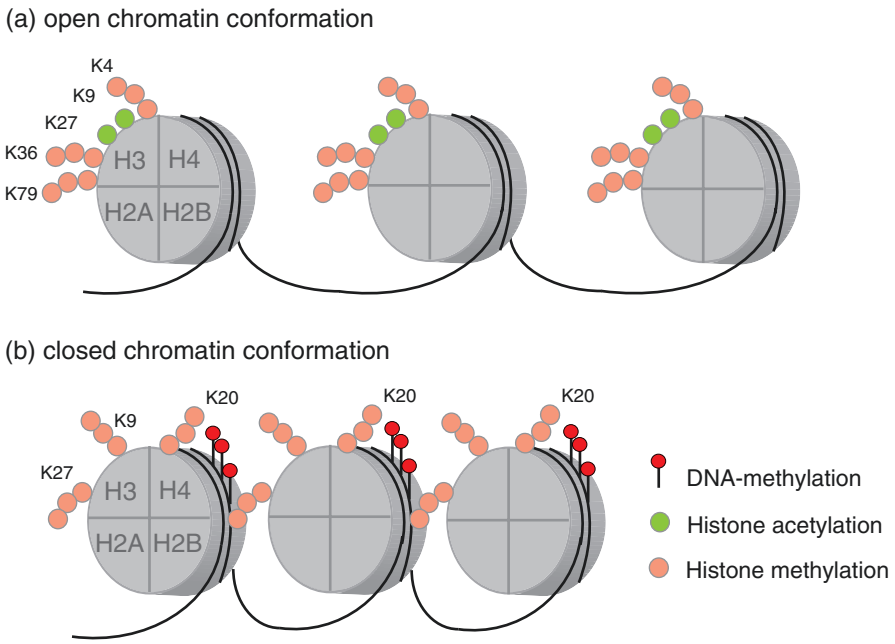

Fig. 12.1 Histone modifications determine open or closed chromatin conformation. (**a**) The acetylation or methylation of specific lysine residues (K4, K9, K27, K36, K79) on histone H3 is associated with transcriptional gene activation and an open chromatin conformation. (**b**) The methylation of the lysine residues K9, K20, and K27 on histone H3 and DNA methylation on CpG islands lead to gene silencing and a closed chromatin conformation

triggered through genetic, environmental, or metabolic factors, the epigenetic alterations can act as an oncogenic stimulus initiating tumor development or accelerating tumor progression [8]. Epigenetic modifications are deposited by "writers," removed by "erasers," and recognized by "readers" (Fig. 12.2). The enzymes are often dysregulated in cancer and are therefore potential targets for an epigenetic-based therapy. The number of epigenetic drugs has highly increased over the last years. The first epigenetic drugs approved by the US Food and Drug Administration (FDA) were the nucleoside analogues 5-azacytidine (5-aza or azacitidine) and 5-aza-2′-deoxycytidine (decitabine), which target DNA methyltransferases (DNMTs) and the histone deacetylase (HDAC) inhibitors vorinostat and romidepsin. In 2014 and 2015, the HDAC inhibitors (HDACi) belinostat and panobinostat were accepted for the indicated diseases, listed in Table 12.1 [9, 10]. Many other epigenetic drugs are currently under investigation in preclinical and clinical trials for various tumor entities (Fig. 12.2). However, the application of these drugs for pancreatic cancer therapies requires a deeper understanding of the epigenetic mechanisms in pancreatic carcinogenesis.

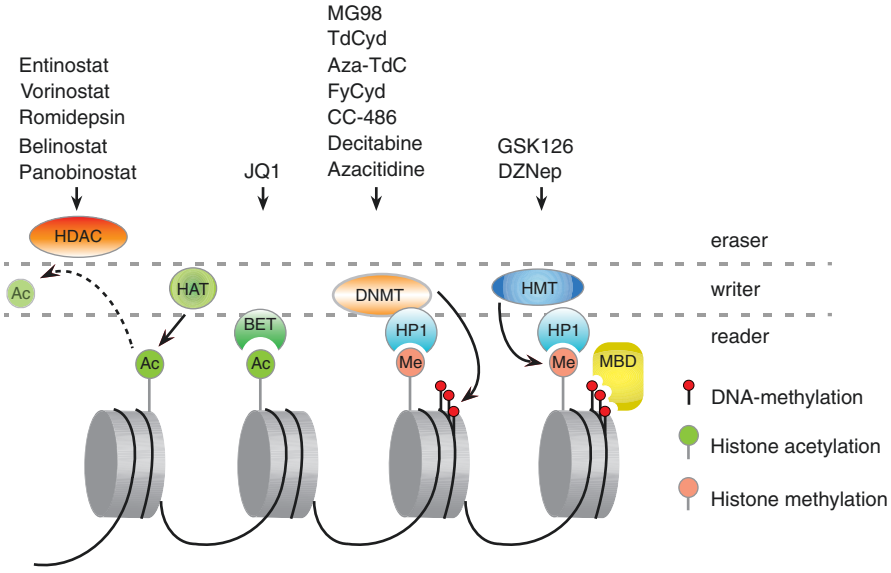

Fig. 12.2 Epigenetic modifiers and their inhibitors. Epigenetic modifications are catalyzed by "writers," such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), and DNA methyltransferases (DNMTs). The marks are removed by "erasers," such as histone deacetylases (HDACs), and recognized by the "readers," bromodomain and extra-terminal (BET) proteins, heterochromatin protein 1 (HP1), and methyl-CpG-binding domain proteins (MBDs). The FDA-approved epigenetic inhibitors are listed in bold; FyCyd, 5-fluoro-2′-deoxycytidine; Aza-TdC, 5-aza-4′-thio-2′-deoxycytidine; TdCyd, 4′-thio-2′-deoxycytidine, DZNep 3-deazaneplanocin

Table 12.1 US Food and Drug Administration (FDA)-approved epigenetic drugs

Epigenetic drug	Classification	Approved year	Proposed indication
Azacitidine	DNMT inhibitor	2004	Myelodysplastic syndromes
Decitabine	DNMT inhibitor	2006	Myelodysplastic syndromes
Vorinostat	HDAC inhibitor	2006	Cutaneous T-cell lymphoma
Romidepsin	HDAC inhibitor	2009	Cutaneous T-cell lymphoma
Belinostat	HDAC inhibitor	2014	Peripheral T-cell lymphoma
Panobinostat	HDAC inhibitor	2015	Multiple myeloma

Modified from Li et al. [10] and updated on the US FDA web page (https://www.fda.gov/Drugs/default.htm)

Preclinical Studies on Epigenetic Alterations

Histone Acetylation

The acetylation and deacetylation of histones is a crucial mechanism for gene regulation and controlled by histone acetyl transferases (HATs) and HDACs, respectively (Fig. 12.2). HATs, such as CREB-binding protein, p300, and KAT2B, catalyze

the acetylation of lysine residues on histone tails and promote gene expression, whereas the removal of the acetyl groups, for example, through class I HDAC1, HDAC2, HDAC3, or class III sirtuin (SIRT) enzymes, leads to transcriptional silencing. Particularly, class I HDACs are extensively studied in different tumor entities, including pancreatic cancer. The overexpression of HDACs in pancreatic cancer is associated with poor patient survival, high proliferation activity, increased tumor grade, and epithelial-to-mesenchymal transition (EMT) demonstrating their oncogenic potential [11]. Recent studies have investigated the acetylation of histone H3 on lysine 27 (H3K27ac), a target of class I HDACs, in more detail. Histone acetylation at gene enhancer regions separated low- and high-grade PDACs and correlated well with a grade-specific gene expression programs. Thus, high levels of H3K27ac were associated with increased activation of epithelial genes in low-grade tumors [5]. Interestingly, another study showed that an H3K27ac-based reprogramming of enhancer elements occurs predominantly in metastatic lesions. It was noted that a gain of H3K27ac at specific gene enhancers promotes PDAC progression and metastatic potential [12]. These data illustrate that the targeted genomic loci of HATs and HDACs are highly dynamic in tumor evolution and phenotypic variants. Notably, histone acetylation can be found either on differentiation genes or on genes driving metastatic mechanisms. These dynamic and contradictory processes highlight the need for further information on epigenetic regulation and targeting, particularly under which conditions the application of epigenetic drugs would result in a clinical benefit.

In preclinical settings, HDACi show a variety of antitumor effects. HDACi promote cell cycle arrest by regulating the expression of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors such as p21. HDACi also induce cell death through the activation of intrinsic and extrinsic apoptotic pathways and inhibit angiogenesis by regulating *HIF1A* and *VEGF* gene expression. Furthermore, some HDACi are able to stimulate the expression of E-cadherin, a potent suppressor of EMT and metastases formation [13]. Besides HDACi, recent experimental data demonstrated that targeting histone acetylation "readers," namely, BET (bromodomain and extra-terminal) proteins, suppresses PDAC development in a pancreatic cancer mouse model (Fig. 12.2). Bromodomain-containing (BRD) proteins, such as BRD2, BRD3, and BRD4, are highly expressed in PDAC and stimulate the expression of the proto-oncogene MYC. Treatment of pancreatic cancer mice with the BET inhibitor JQ1 in combination with the HDACi vorinostat resulted in significant longer overall survival and decreased tumor volume compared to JQ1 monotherapy, revealing promising results for clinical translation [14].

Histone Methylation

While histone acetylation is associated with gene expression, mono-, di-, or trimethylation of lysine residues on histone tails can have different effects. Methylation of H3 lysine 4 (H3K4me), lysine 36 (H3K36me), and lysine 79 (H3K79me) marks

active gene loci, whereas methylation of H3 lysine 9 (H3K9me), lysine 27 (H3K27me), and H4 lysine 20 (H4K20me) correlates with gene silencing (Fig. 12.1) [15]. So far, over 50 lysine methyltransferases (KMTs) have been identified, subdivided in DOT1-like proteins and the SET domain-containing protein group [16]. The polycomb repressive complex (PRC) 2 subunit, enhancer of zeste (EZH2), which belongs to the SET domain-containing KMTs, catalyzes the repressive histone modification H3K27me (Fig. 12.2). In experimental approaches, several EZH2 inhibitors (EZH2i) demonstrate therapeutic potential for cancer treatment. The EZH2i 3-deazaneplanocin (DZNep) attenuates EMT and sensitizes pancreatic cancer cells for standard chemotherapy [17, 18]. A more potent EZH2i, GSK126, was tested in a large panel of cancer cell lines, including pancreatic cancer cells [19]. However, it should be noted that in a subset of cells, a loss of H3K27me after GSK126 treatment entailed an increase of transcriptionally active H3K27ac levels targeting preferentially oncogenes, which trigger tumor progression. The oncogenic effect was abandoned after additional BET inhibition [19]. Changing the global epigenetic landscape in a non-stratified manner may cause unintended effects and therapeutic controversies. These data highlight the need for a greater understanding of the molecular mechanisms after epigenetic drug treatment and a possible application of combination therapies for treatment success.

DNA Methylation

Cytosine residues in CG dinucleotide clusters, so-called CpG islands, are targets for DNA methylation. Sixty percent of the human gene promoters are associated with CpG islands and are mostly unmethylated, whereas CpG regions in repetitive elements and heterochromatin are heavily methylated [15]. The DNA methyltransferases 3A and 3B (DNMT3A and DNMT3B) regulate de novo methylation during embryogenesis, whereas DNMT1 catalyzes the methylation of the newly synthesized DNA strand after replication [16]. Methyl-CpG-binding domain proteins (MBDs) are epigenetic "readers" and recruit epigenetic repressor complexes, which mediate further chromatin compaction (Fig. 12.2). Genome-wide alterations of DNA methylation are a common event in tumorigenesis. Malignant cells exhibit an epigenetic silencing of tumor suppressor genes, such as *CDKN2A* (p16), through promoter hypermethylation, whereas the global genome is hypomethylated fostering chromosomal instability and oncogenicity [8]. Early in PDAC development, precursor lesions, such as pancreatic intraepithelial neoplasia (PanINs), show a hypermethylation of *CDKN2A* and other tumor suppressor genes [20]. Further methylated genes, regulating key signaling pathways in cell differentiation, were identified in PDAC patient samples [21] showing the significance of aberrant DNA methylation for pancreatic carcinogenesis.

The nucleoside analog 5-aza was the first synthesized epigenetic drug. It intercalates into the DNA and inhibits DNMT through permanent binding after DNA replication [22]. The treatment of pancreatic cancer cell lines with 5-aza significantly

impaired cell proliferation and induced apoptosis in a time- and concentration-dependent manner. Moreover, 5-aza treatment decreased the expression of key molecules of the Wnt signaling pathway, such as β-catenin and MYC, which play an important role in tumor cell migration and metastasis [23]. Further studies have noted that 5-aza has the capability to sensitize cancer cells for chemo- or radiotherapy increasing the effectiveness of antitumor therapy [22, 24].

Although preclinical studies have made significant progress in understanding epigenetic changes in pancreatic cancer, there is still a lack of knowledge of the highly dynamic epigenetic mechanisms and their regulation in pancreatic tumor development and progression. Pharmacologic inhibition of histone-modifying enzymes or DNA methyltransferases has shown promising effects in experimental approaches. However, elucidating the conditions for epigenetic drug treatment and identifying patients who will profit from a certain epigenetic therapy will be the challenge for translating the experimental data into the clinical practice.

Clinical Trials Targeting Epigenetic Alterations

For many years, gemcitabine-based therapies have been the standard of care for patients suffering from PDAC. However, median overall survival stayed below 3 years (35.0 months) after adjuvant chemotherapy with gemcitabine. Admission of the modified FOLFIRINOX combination therapy (folinic acid + 5-fluorouracil + irinotecan + oxaliplatin) was able to raise the overall survival to 54.4 months at the price of increased toxicity [25]. Hence, there is a great need for new therapeutic strategies. Several phase I and II clinical trials address the tolerability and potency of epigenetic drugs as mono- or combination therapy in the setting of advanced disease refractory to standard therapy.

Vorinostat, also known as suberanilohydroxamic acid (SAHA), is an inhibitor of class I and II HDACs and was approved for the treatment of cutaneous T-cell lymphoma in 2006 (Table 12.1). Due to its broad effects on cell cycle arrest, apoptosis, angiogenesis, and tumor microenvironment, vorinostat is currently under investigation in several clinical studies for the treatment of solid tumors (Table 12.2). Although monotherapies of HDACi show a high efficiency in hematologic malignancies, the antitumor effects for solid cancers are mostly unsatisfactory. Thus, combination therapies of epigenetic inhibitors with classical chemotherapeutic agents or small-molecule inhibitors are enrolled for clinical trials [26]. The combination of vorinostat with marizomib, a proteasome inhibitor, was tested in a phase I clinical trial (NCT00667082) in 22 patients with solid cancers, including pancreatic cancer. The data showed stable disease in 61% of evaluable patients [27]. Similarly, the combinatory treatment of vorinostat with the proteasome inhibitor bortezomib in patients with advanced solid tumors (NCT00227513) revealed a stable disease in patients with sarcoma, colorectal cancer, and gastrointestinal stroma tumor (GIST), whereas pancreatic cancer patients were unaffected. The most common adverse events were hematologic and gastrointestinal-related toxicities [28]. Another phase

Table 12.2 Overview of HDAC inhibitors in clinical trials for pancreatic cancer

HDACi	Combination therapy	Phase	Patients	Dosage schedule (HDACi)	Outcome	Serious adverse events	NCT number
Vorinostat	Marizomib	I	22	300 mg/day	SD: 11/18 (61%)	Thrombocytopenia, nausea/vomiting	00667082
	Bortezomib	I	66	Dose escalation (100–300 mg/twice daily)	MTD: 1.3 mg/m^2 iv SD: 5/22 (23%)	Thrombocytopenia, fatigue, increased ALT, diarrhea, nausea	00227513
	Sirolimus	I	249	Dose escalation (100–400 mg/day)	PR: 2/61 (3%) SD: 2/61 (3%) PFS: 9 weeks	Thrombocytopenia, anemia, fatigue, mucositis	01087554
	5-FU, radiation	I/II	10	Varying doses/day, 6 weeks	OS: 6/9 (66.7%) RR: 6/9 (66.7%) PFS: 9.375 months	Hematologic and gastrointestinal, fatigue	00948688
	Capecitabine, radiation	I	21	Dose escalation (100–400 mg/day during radiation and Mon–Fri 2 weeks after radiation)	MTD: 400 mg/day SD: 19/21 (90%)	Lymphopenia, thrombocytopenia, nausea, vomiting, diarrhea	00983268
Belinostat	Carboplatin, paclitaxel	I	23	Dose escalation (600–1000 mg/m^2 Mon–Fri)	PR: 2/23 (9%) SD: 7/23 (30%)	Hematologic	00873119
Panobinostat	Gemcitabine	I	17	Dose escalation	PR: 1/17 (6%) SD: 8/17 (47%)	Hematologic, gastrointestinal, fatigue, rash	00550199
	Bortezomib	II	7	20 mg/3 times a week, 2 weeks	PFS: 2.1 months PD: 5/7 (71%)	Thromboembolic events (2/7), gastrointestinal, fever, dehydration	01056601
Entinostat	Nivolumab	II	54	5 mg 2 lead-in doses 5 mg/week	*Recruiting*		03250273

MTD maximum tolerated dose, *OS* overall survival, *PD* progressive disease, *PFS* progression-free survival, *PR* partial response, *RR* response rate, *SD* stable disease

I dose-escalating study (NCT01087554) evaluated the synergistic effects of the mTOR inhibitor sirolimus and vorinostat in 70 patients, including two patients with pancreatic cancer. They observed two partial responses in patients with refractory Hodgkin lymphoma and perivascular epithelioid cancer. Two patients with hepatocellular carcinoma and fibromyxoid sarcoma had stable disease for at least 12 months. The most common dose-limiting toxicities included grade 4 thrombocytopenia and grade 3 mucositis [29]. A phase I/II clinical trial (NCT00948688) investigated the effects of the antimetabolite 5-fluorouracil (5-FU) in combination with vorinostat and radiation therapy. Ten patients with pancreatic cancer received varying doses of vorinostat for 6 weeks to uncover the highest dosage for safe administration. One year after the study enrollment, 66.7% of the patients were still alive, and the overall response rate was 66.7%. Progression-free survival after 2 years was 9.4 months. Here, the most common serious adverse events were hematologic and gastrointestinal side effects, but no dose-limiting toxicities were observed (unpublished data from https://clinicaltrials.gov/). Furthermore, a phase I dose-escalating trial (NCT00983268) from 2009 to 2012 tested the efficacy of vorinostat and capecitabine as radiosensitizers in 21 patients with pancreatic cancer. Capecitabine was administered at a dosage of 1000 mg on the days of radiation, whereas vorinostat was given orally on the days of radiation and continued for 2 weeks on Monday through Friday after completing the radiation therapy. The maximum tolerated dose of vorinostat was 400 mg/day. They reported thrombocytopenia, nausea and vomiting, diarrhea, and dehydration as dose-limiting toxicities. The most common study-related adverse events with a grade ≥ 3 were lymphopenia (67%) and nausea (14%). Notably, 90% of the patients had stable disease and 33% of patients initially classified as borderline-resectable underwent R0 or R1 resections. The median overall survival in the study was 1.1 years [30]. Even though only a small number of patients were investigated, the study results were encouraging, since advanced PDAC patients rarely show a radiosensitive response.

Belinostat is a pan-HDACi and was approved for the treatment of relapsed or refractory peripheral T-cell lymphoma in 2014 (Table 12.1). Preclinical studies have shown that belinostat inhibits cell growth and initiates apoptosis and cell cycle arrest in a dose-dependent manner. Furthermore, belinostat inactivates downstream signaling of the PI3K-mTOR pathway and blocks hypoxia-induced pro-tumorigenic mechanisms [31, 32]. Along with several experimental approaches to further determine pharmacokinetics and safety of belinostat, first phase I clinical trials are conducted in patients with solid tumors (Table 12.2). In a phase I dose-escalating trial (NCT00873119), 23 patients with solid tumors, including three cases with pancreatic cancer, received 600–1000 mg/m^2 belinostat per day on day one to five of each cycle in combination with carboplatin and/or paclitaxel. No dose-limiting toxicities were observed. Grade 3 adverse events included neutropenia (30%), leukopenia (22%), and thrombocytopenia (13%). Two patients with metastatic pancreatic and metastatic rectal cancer showed a partial response of 7 and 9 months, respectively. Seven patients showed stable disease for ≥6 months (range 6–29 months) [33].

Another pan-HDACi, approved for hematologic malignancy (multiple myeloma) (Table 12.1), which is currently under clinical testing for the treatment of solid

tumors, is *panobinostat* (Table 12.2). In contrast to other HDACi, panobinostat has a longer elimination time and shows an increased potency against hyperacetylating histone proteins [34]. Therefore, intermittent dosing schedules are needed to reduce common dose-limiting toxicities of HDACi. In a phase I clinical study (NCT00550199), 17 patients with solid tumors received oral panobinostat over five different dose levels (continuous or intermittent dosing) in combination with intravenous gemcitabine treatment. Nevertheless, grade four hematologic side effects, notably thrombocytopenia and neutropenia, occurred at all dose levels and required multiple changes in the study protocol. Other adverse events included anorexia, constipation, diarrhea, fatigue, nausea, vomiting, and rash. Besides one unconfirmed partial response in a patient with ovarian cancer, eight patients including one patient with pancreatic cancer had stable disease (median duration six cycles) [35]. However, another clinical study (NCT01056601) utilizing a combination of panobinostat and bortezomib showed progressive disease in >70% of the participants. More preclinical studies are urgently needed to identify panobinostat treatment strategies and combinatory agents improving patient outcome.

Several other new HDACi are currently under investigation in various combinatory trials. For example, an ongoing clinical study (NCT03250273) is testing the safety and efficiency of *entinostat*, a class I HDACi, in combination with nivolumab, an IgG4 monoclonal antibody, on patients with pancreatic cancer or with tumors of the biliary tract (Table 12.2). The first results are eagerly awaited.

Azacitidine (5-azacytidine) and decitabine (*5-aza-2′-deoxycytidine)* are DNMT inhibitors (DNMTi) and approved for the treatment of myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CMML), respectively (Table 12.1). The nucleoside analogues share structural similarities with cytidine and are incorporated into the DNA during the replication of cancer cells, which causes direct cytotoxicity [22]. In several clinical trials, DNMTi are investigated as mono- or combinatory therapy (Table 12.3). The combination of 5-aza and gemcitabine was tested in a phase I dose escalation study on nine patients with advanced pancreatic cancer (NCT01167816). Furthermore, two phase II studies with 5-aza and pembrolizumab, an immune checkpoint inhibitor, are ongoing (NCT03264404, NCT01845805). Due to the rapid deamination of (deoxy-) cytidine to (deoxy-) uridine, cytidine deaminase inhibitors like tetrahydrouridine (THU) are co-administered with cytidine analogues [36]. In a phase I clinical trial (NCT00359606), 58 patients with refractory solid tumors received the cytidine analogue *5-fluoro-2′-deoxycytidine* (FyCyd). The DNMTi was administered intravenously on days 1–5 every 3 weeks or on days 1–5 and 8–12 every 4 weeks together with a fixed dose of 350 mg/m^2/day THU. In the 3-week schedule, no dose-limiting toxicities were observed. In the 4-week schedule, one dose-limiting grade 3 colitis was reported during the first cycle. The MTD was defined as 134 mg/m^2/day. Fifty percent of the patients (20/40) included in this study reached stable disease as best outcome (1.4–13.3 months). One partial response was reported in a heavily pretreated breast cancer patient (>90% decrease in tumor size) and maintained for 15.2 months. Unfortunately, no patients with pancreatic cancer were included in this study [37]. A second phase I trial (NCT01534598) investigates the effects of a

Table 12.3 Overview of DNMT inhibitors in clinical trials for solid tumors

DNMTi	Combination therapy	Phase	Patients	Dosage schedule (DNMTi)	Outcome	NCT number
5-Azacytidine (azacitidine)	Gemcitabine	I	9	Dose escalation, 5 consecutive days each 4-week cycle	*Terminated*	01167816
	Pembrolizumab	II	31	50 mg/m^2 subcutaneous daily, 5 days each 4-week cycle	*Recruiting*	03264404
		II	80	300 mg daily on days 1–21 of each 4-week cycle	*Recruiting*	01845805
5-Fluoro-2′-deoxycytidine (FyCyd)	Tetrahydrouridine (THU)	I	58	Dose escalation, days 1–5 of each 3-week cycle or days 1–5 and 8–12 of each 4-week cycle	MTD: 134 mg/m^2 SD: 20/40 (50%) PR: 1/40 (3%)	00359606
	Tetrahydrouridine (THU)	I	68	Dose escalation, days 1 to 3 and 8 to 10 of a 3-week cycle, oral administration	*Recruiting*	01534598
CC-486	Carboplatin, Nab-paclitaxel	I	169	Dose escalation	PR: 5/57 (9%) RR: 6/57 (11%)	01478685
5-Aza-2′-deoxycytidine (decitabine)	Gemcitabine	I	42	Dose escalation starting at 0.1 mg/kg subcutaneously, two times a week for 3 weeks of a 4-week cycle	*Recruiting*	02959164
	Tetrahydrouridine (THU)	I	13	Starting dose by weight (10–20 mg daily) two times a week on consecutive days	*Active*	02847000
MG98		I	20	Dose escalation (40–360 mg/m^2), 21 consecutive days of 4-week cycle	No changes in DNMT1 levels	
		I	33	Dose escalation, 7 consecutive days of a 2-week cycle	PR: 1/33 (3%)	

MTD maximum tolerated dose, *PR* partial response, *RR* response rate, *SD* stable disease

different dosage schedule of FyCyd and THU in 68 patients with advanced solid tumors. Moreover, pharmacokinetics and efficacy of an oral formulation of 5-azacytidine (*CC-486*) were explored in patients with relapsed or refractory solid tumors in a phase I clinical trial (NCT01478685). The first part of the trial included a dose escalation study of CC-486 alone or in combination with carboplatin or nab-paclitaxel in 57 patients. In the second part of the study, 112 patients were assigned to treatment arms according to the tumor type and received CC-486 at the recommended dose alone or in combination with carboplatin or nab-paclitaxel. Thus, patients with pancreatic cancer received 200 mg CC-486 orally for 2 weeks and 100 mg/m^2 nab-paclitaxel intravenously on days 1 and 14. The objective response rate in the first part of the study was 10.5% (6/57), and five partial responses were reported in the study arms with CC-486 monotherapy and CC-486 in combination with nab-paclitaxel. In the second part, partial responses were detected in patients with bladder cancer, ovarian cancer, non-small cell lung carcinoma (NSCLC), nasopharyngeal carcinoma (NPC), and other virus-associated tumors (OVAT). Disease control rates reached 45.8% in pancreatic cancer. The most common adverse event with grade ≥ 3 in all study arms was neutropenia. The study showed promising effects and partial response rates for several tumor entities; however, the CC-486 combination therapy with carboplatin or nab-paclitaxel did not improve the overall response in comparison to the CC-486 monotherapy [38].

New treatment strategies for solid tumors also implement the use of the DNMTi *decitabine* (Table 12.3). Two ongoing phase I clinical trials aim at determining the maximum tolerated dose, as well as outcome and dose-limiting toxicities in combination with the classic chemotherapeutic agent gemcitabine (NCT02959164) or with the cytidine deaminase inhibitor THU (NCT02847000). Most clinical studies for DNMTi are phase I trials testing drug safety in patients with advanced solid tumors. Thus, different dosage regimens of new agents, such as *5-aza-4′-thio-2′-deoxycytidine* (Aza-TdC) (NCT03366116) and *4′-thio-2′-Deoxycytidine* (TdCyd) (NCT02423057), are elucidated for safe drug administration. A second-generation DNMT1 inhibitor, which was tested in clinical trials, is *MG98*. This is a phosphorothioate antisense oligonucleotide, which inhibits DNMT1 translation with a high specificity [39]. Fourteen patients with solid cancers received MG98 in a dose-escalating phase I study. MG98 was administered intravenously for 21 consecutive days, followed by 1-week rest period. A significant number of patients experienced dose-limiting transaminase elevations. Other dose-limiting toxicities included fatigue, anorexia, and thrombocytopenia. Although the mean plasma drug concentrations of MG98 were ten times higher than the IC_{50} values determined in vitro (50–70 nM), no significant changes in DNMT1 levels were observed [40]. Later on, another phase I dose escalation clinical trial investigated the effects of MG98 on DNMT1 expression in 33 patients with advanced solid tumors, including four patients with tumors of the pancreas or biliary system. Here, MG98 was administered by continuous intravenous infusion over 7 days in a 2-week cycle. The most common observed drug-related toxicities were grade 3 transaminitis and grade 3 thrombocytopenia. Other side effects included fatigue, headaches, myalgia, and nausea. One patient with esophageal cancer achieved a partial response, and

another patient with GIST showed prolonged disease stabilization for more than 3 years [39].

Although DNMTi are tested in a variety of clinical studies with different co-medications, only one study protocol using a combination of FyCyd and THU [35] showed promising results in solid tumors. However, this study did not include patients with pancreatic cancer. Hence, there is a great need to include more patients with advanced pancreatic cancer in clinical trials investigating safety and efficacy of DNMTi.

Conclusion

Hematologic malignancies have been successfully treated with epigenetic drugs, such as vorinostat or azacitidine, for several years. Although more and more clinical studies exhibited promising effects of epigenetic drugs in solid tumor therapy regimens, there are many aspects not uncovered yet transferring experimental data into a successful clinical approach. Notably, broad antitumorigenic effects of HDACi and DNMTi were detected in experimental settings, but most clinical trials testing epigenetic treatment strategies in pancreatic cancer patients demonstrated rather disappointing results on overall survival and response rates. One aspect might be a required stratification of pancreatic cancer patients according to their epigenetic landscape to identify those patients who would benefit from an epigenetic treatment. Furthermore, the molecular mechanisms affected by epigenetic drugs are barely understood. The influence of unknown regulators, activated in response to epigenetic treatment, might limit the efficiency of epigenetic therapies. Moreover, the dynamic nature of epigenetic modifications targeting either tumor suppressor genes or oncogenes makes the application of epigenetic drugs sometimes unpredictable. An additional complication of epigenetic remodeler inhibition is the complete blockage of their enzymatic activity without the possibility to rescue the physiological function. Consequently, more preclinical and clinical studies are needed to investigate the molecular mechanisms controlled by epigenetic modifiers. Revealing the functional consequences of epigenetic drug treatment, under the consideration of various pancreatic cancer subtypes, will be a future challenge. Nevertheless, several phase I clinical trials were able to determine the maximum tolerated dose for HDACi, DNMTi, and other epigenetic inhibitors, building a foundation for further epigenetic treatment strategies.

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Chapter 13
Targeting Metabolism

Yoshiaki Sunami

Understanding Metabolic Reprogramming to Improve Therapeutic Strategies in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with an unfavorable outcome and is projected to become the second deadliest cancer by 2030, and currently the overall 5-year survival rate is less than 7% [1, 2]. PDAC arises through multistage genetic and histological progression from precursors such as pancreatic intraepithelial neoplasia (PanIN). Activating mutations in the *KRAS* oncogene are observed in over 90% of PDAC patients, and using genetically engineered mouse models, it has been demonstrated that *KRAS* mutations influence tumor initiation, progression, and maintenance [3, 4]. Tumorigenesis is dependent on the reprogramming of cellular metabolism which can be a consequence of oncogenic mutations. In line with this, a profound rewiring of metabolic pathways involved in, e.g., glucose, glutamine, and lipid metabolisms, is activated downstream of oncogenic *KRAS* [5]. In general, metabolic reprogramming has now been recognized as a hallmark of cancer [6]. Cancer cells manipulate metabolisms to keep generating their own cellular components such as DNA, proteins, and lipids for maintaining rapid cell growth. Understanding and identification of metabolic reprogramming strategies of individual cancers could uncover novel potential personalized targets. This chapter provides a background of cancer metabolism focusing on glucose, glutamine, acetate, and lipid metabolism and targeting strategies for modulating enzymes/factors involved in key metabolic pathways.

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Glucose Metabolism and Pentose Phosphate Pathway in Pancreatic Cancer

Warburg Effect and Reprogramming of Glucose Metabolism: The Role of Gene Mutations

A pioneer of the study of cancer metabolic reprogramming, Otto Heinrich Warburg made a striking discovery known as Warburg effect that many cancer cells preferentially convert glucose into lactate (fermentation) rather than respiration – transporting pyruvate into mitochondria and converted it into acetyl-CoA for subsequent ATP production via the citric acid cycle and electron transport chain – even in the presence of oxygen [7–10]. Glycolysis, a metabolic pathway that converts glucose into pyruvate (and lactate) in the cytoplasm, is a sequence of ten enzyme-catalyzed reactions (Fig. 13.1). The three reactions converting glucose into glucose 6-phosphate by hexokinase (HK), fructose 6-phosphate into fructose 1,6-bisphosphate by phosphofructokinase (PFK), and phosphoenolpyruvate into pyruvate by pyruvate kinase are key steps. Oncogenic $KRAS^{G12D}$ plays a role in upregulating gene expression of the glucose transporter (GLUT) *Slc2a1* (SLC: solute carrier), *Hk1*, *Hk2*, and *Pfk1* as well as *Ldha* coding lactate dehydrogenase (LDH), an enzyme for converting pyruvate to lactate. Concomitantly oncogenic KRAS enhances glucose uptake and lactate production in a pancreatic cancer mouse model [11]. The transcription factor p53 is recognized as a key tumor suppressor and also frequently mutated in human tumors. Missense mutations such as R175H, R248Q, and R273H not only result in loss of the tumor suppressive function of p53 but also in oncogenic functions that promote invasion, metastasis, proliferation, and cell survival [12]. Mutation of p53 also enhances glucose uptake by GLUT1 translocation, glycolytic rate, and lactate production in R172H mutant-expressing p53 in murine cancer cells or fibroblasts (R172H is equivalent to human R175H) [13]. Deficiency of another tumor suppressor gene, *SMAD4*, increases GLUT1 levels and lactate production in cancer cells [14]. *KRAS*, *TP53*, *CDKN2A,* and *SMAD4* are the most prevalent genetic mutations in pancreatic cancer [1]; yet these genes are currently not druggable. However, targeting glucose metabolic reprogramming may provide a selective mechanism for eliminating cancer cells.

Targeting Enzymes and Factors Involved in Glucose Metabolism

Inhibition of GLUT, especially GLUT1 expression, can be an option to halt the proliferation of cancers. A small-molecule GLUT1 inhibitor WZB117 has been shown to block glucose uptake and tumor growth in a tumor xenograft model [15, 16]. Furthermore, WZB117 administration inhibits tumor initiation after implantation of cancer stemlike cells derived from pancreatic cancer cells without causing adverse events in host mice [17]. Overexpression of GLUT1 correlates with poor

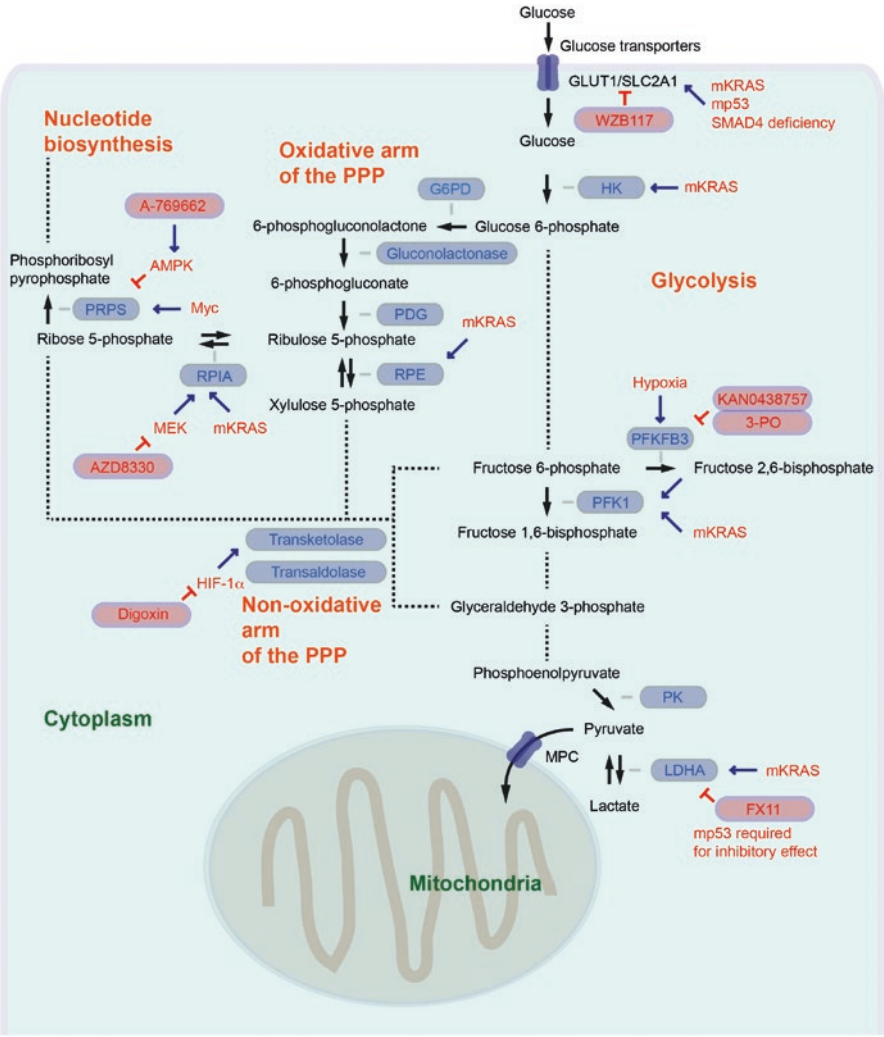

Fig. 13.1 Regulation of glycolysis, pentose phosphate pathway, and nucleotide biosynthesis. AMPK AMP-activated protein kinase, G6PD glucose 6-phosphate dehydrogenase, HIF hypoxia-inducible factor, HK hexokinase, LDH lactate dehydrogenase, mKRAS mutant KRAS, mp53 mutant p53, MPC mitochondria pyruvate carrier, PFK phosphofructokinase, PGD 6-phosphogluconate dehydrogenase PK pyruvate kinase, PPP pentose phosphate pathway, PRPS phosphoribosylpyrophosphate synthetase, RPE ribose 5-phosphate-3-epimerase, RPIA ribose 5-phosphate isomerase A, SLC solute carrier

overall survival of several solid tumors [18], and high GLUT1 expression is also suggested to predict shorter overall survival in patients with pancreatic cancer [19].

In the mammalian glycolytic pathway, PFK1 is rate-limiting and the most important control element. When PFK1 is inactive, the concentration of fructose

6-phosphate rises, and in equilibrium, the level of glucose 6-phosphate also rises. Hexokinase, another key enzyme in the glycolytic pathway, is allosterically inhibited by glucose 6-phosphate; therefore, PFK1 inhibition leads to the inhibition of hexokinase. Activity of PFK1 is stimulated by fructose 2,6-bisphosphate, which is derived from fructose 6-phosphate catalyzed by PFK2. There are four PFK2 isoforms (PFKFB1–4), and PFKFB3 is highly expressed in many types of human cancer including pancreatic cancer [20]. Expression of PFKFB3 can also be regulated by hypoxia [21]. PFKFB3 also regulates angiogenesis and vessel branching [22] and can be an emerging anticancer target. In this line, KAN0438757 has been considered as a selective PFKFB3 inhibitor, and treatment with this inhibitor radiosensitizes cancer cells [23]. Another PFKFB3 blocker 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) reduces orthotopically implanted pancreatic cancer cell development [24], suggesting that targeting PFKFB3 can be an option for pancreatic cancer treatment.

In the late step in glycolysis, pyruvate kinase plays an important role as catalyzing the last physiological irreversible reaction to produce pyruvate. In mammals, there are four pyruvate kinase isoforms encoded by two genes: isoforms PKL and PKR are derived from the PKLR gene, and PKM1 and PKM2 are derived from the PKM gene through alternative splicing. The amino acid differences in PKM2 result in a fructose 1,6-bisphosphate-binding pocket for positive allosteric regulation [25]. Activation of PFK1 (for producing fructose 1,6-bisphosphate) can therefore not only regulate hexokinase activity but also PKM2 activity. PKM2 is expressed during embryogenesis, regeneration processes, and in cancer, suggesting that PKM2 activity is important in actively proliferating cells [25]. Orthotopically implanted cancer cells expressing PKM2 support tumorigenesis, whereas cells expressing PKM1 reduce tumorigenicity, suggesting that the PKM2 splice isoform is important for cancer metabolism and tumor growth [26]. On the contrary, in some studies activation of PKM2 can inhibit cancer cell proliferation [27, 28]. Furthermore, conditional deletion of PKM2 in a pancreatic cancer mouse model (oncogenic $KRAS^{G12D}$ expression and p53 deletion) does not affect mouse survival, tumor weight, or tumor histology [29]. Therefore, targeting PKM2 might not be suitable for pancreatic cancer treatment and needs further investigation.

Pyruvate is a key metabolite in the network of metabolic pathways. Pyruvate in the cytoplasm can be converted into alanine by alanine aminotransferase (ALT) or transported into the mitochondria via mitochondria pyruvate carrier (MPC) and converted there into oxaloacetate by pyruvate carboxylase for gluconeogenesis or converted into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex for the citric acid cycle. Pyruvate decarboxylase catalyzes a reaction converting pyruvate into acetaldehyde in the cytoplasm and mitochondria. Cancer cells however preferentially convert pyruvate into lactate, which is catalyzed by LDH. LDH is a tetramer of two subunits LDHA and LDHB, which assemble into five different combinations [30]. LDHA has a higher affinity for pyruvate than LDHB, and elevated levels of LDHA are a hallmark of many cancer types; hence targeting LDHA can be a promising strategy for cancer therapeutics. Consistently, FX11 (3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid), a small-molecule

inhibitor of LDHA, inhibits the progression of pancreatic cancer xenografts [31]. Interestingly, the inhibitory effect of FX11 requires mutant p53, and FX11 treatment does not inhibit tumor progression of patient-derived PDAC xenografts without p53 mutation [32], suggesting that targeting LDHA in pancreatic cancer can be an attractive stratification option since drug responsiveness in PDAC patients may depend on the genetic status.

Pentose Phosphate Pathway: Helper of Cancer's Anabolic Demands

The pentose phosphate pathway (PPP) is another pathway in the cytoplasm for glucose catabolism starting from glucose 6-phosphate. The major function of the PPP is not energy production, but generating extramitochondrial nicotinamide adenine dinucleotide phosphate (NADPH), which is required for fatty acid synthesis and for scavenging reactive oxygen species (ROS). The PPP also supports the synthesis of ribonucleotides. The PPP is divided into two parts, namely, the oxidative arm and non-oxidative arm. The oxidative arm is initiated by conversion of glucose 6-phosphate to 6-phosphogluconolactone by glucose 6-phosphate dehydrogenase (G6PD), which is converted into 6-phosphogluconate by gluconolactonase and further converted into ribulose 5-phosphate by 6-phosphogluconate dehydrogenase (PGD). In the non-oxidative phase of the PPP, ribulose 5-phosphate is either reversibly catalyzed by ribose 5-phosphate isomerase A (RPIA) for producing ribose 5-phosphate or reversibly catalyzed by ribose 5-phosphate-3-epimerase (RPE) for producing xylulose 5-phosphate [33]. Ribose 5-phosphate is converted by phosphoribosylpyrophosphate synthetase (PRPS) to phosphoribosyl pyrophosphate, which serves as the backbone for nucleotide synthesis. Oncogenic $KRAS^{G12D}$ upregulates *RPIA* and *RPE* gene expression in murine primary cells of a pancreatic cancer model with oncogenic $KRAS^{G12D}$ and p53 deficiency. Knockdown of *Rpia* or *Rpe* genes in primary cells reduces the flux of glucose into DNA/RNA synthesis and xenograft pancreatic tumor growth [11], and knockdown of *Rpia* gene inhibits human PDAC cell growth [34]. Ribose 5-phosphate and xylulose 5-phosphate in the non-oxidative branch of the PPP can also be reversibly catalyzed by transketolase and aldolase to fructose 6-phosphate or glyceraldehyde 3-phosphate, which can be utilized in the glycolysis [33]. Vice versa, fructose 6-phosphate and glyceraldehyde 3-phosphate in the glycolytic pathway can be incorporated into the PPP pathway, and many cancer cells generate ribose 5-phosphate through the non-oxidative branch of the PPP for de novo nucleotide biosynthesis [35]. Fructose induces transketolase flux and proliferation of pancreatic cancer cells [36]. High fructose intake has been suggested to be associated with increased pancreatic cancer risk [37]. A key regulator of the non-oxidative branch of the PPP is hypoxia-inducible factor (HIF)-1α which increases the carbon flux into the PPP [35], and HIF-1α directly regulates transketolase gene expression [38]. Taken together, the PPP especially the

non-oxidative arm plays an important role in de novo nucleotide biosynthesis, and directly or indirectly targeting enzymes and factors involved in the PPP is a promising therapeutic strategy against pancreatic cancer.

Targeting Enzymes and Factors Involved in the Pentose Phosphate Pathway and Nucleotide Synthesis

Oncogenic $KRAS^{G12D}$ reprograms metabolism of the PPP in PDAC through MAPK and Myc pathways [11, 34]. Myc has been further shown to control PRPS2, but not PRPS1, and functional loss of PRPS2 delays Myc-dependent tumor initiation [39]. Since KRAS and Myc are currently not druggable, targeting RPIA, RPE of the non-oxidative branch of the PPP, as well as targeting PRPS2 in the nucleotide biosynthesis pathway can be considered as therapeutic options. Inhibitors of RPIA, RPE, or PRPS remain largely undiscovered. Especially selective PRPS2 inhibitors are challenging to identify, since PRPS2 shares more than 97% amino acid identity with the PRPS1 [40]. So far, pharmacological inhibitors of effector pathways on cancer metabolism have been used. For example, treatment with the MEK inhibitor AZD8330 decreases *Rpia* gene expression in murine primary cells of a pancreatic cancer model with oncogenic $KRAS^{G12D}$ and p53 deficiency [11]. AMP-activated protein kinase (AMPK) phosphorylation leads to conversion of PRPS hexamer to monomer resulting in inhibition of nucleotide synthesis in cancer cells (AMPK activator: A-769662) [41]. Digoxin is an HIF-1α synthesis inhibitor [42], and targeting HIF-1α leads to reduction of transketolase gene expression and improved gemcitabine sensitivity in pancreatic cancer cells [38]. MEK/MAPK, AMPK, and HIF-1α regulate not only the PPP and/or nucleotide biosynthesis. However, reprogramming the reprogrammed metabolism of the PPP and nucleotide biosynthesis in cancer by modulating effectors is also a promising targeting strategy.

Lipid Metabolism in Pancreatic Cancer

Fatty Acid Synthesis as an Entrance of Lipid Metabolism and Critical for Cancer Cell Proliferation

The most prominent metabolic alteration is known as the Warburg effect. However, cancer cells manipulate many other metabolic pathways for building up their own cellular components. Especially, activating lipid synthesis is highly important for cancer cells, because lipids such as phospholipid bilayers are fundamental structural components enabling cellular proliferation. It has been shown that extracellular lipids can sufficiently stimulate pancreatic cancer cell proliferation [43]. However, in a wide variety of tumors, de novo synthesis of fatty acids (FAs) is activated

irrespective of the levels of circulating lipids. In contrast to normal cells, cancer cells may gain more than 93% of triacylglycerol FAs via de novo synthesis [44]. In the first step of FA synthesis, cytoplasmic acetyl-CoA is generated from citrate by ATP-citrate lyase (ACLY) and then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA and acetyl-CoA are coupled to the acyl-carrier protein (ACP) domain of the multienzyme protein fatty acid synthase (FASN) (Fig. 13.2). Via repeated condensations of acetyl groups by the FASN in an

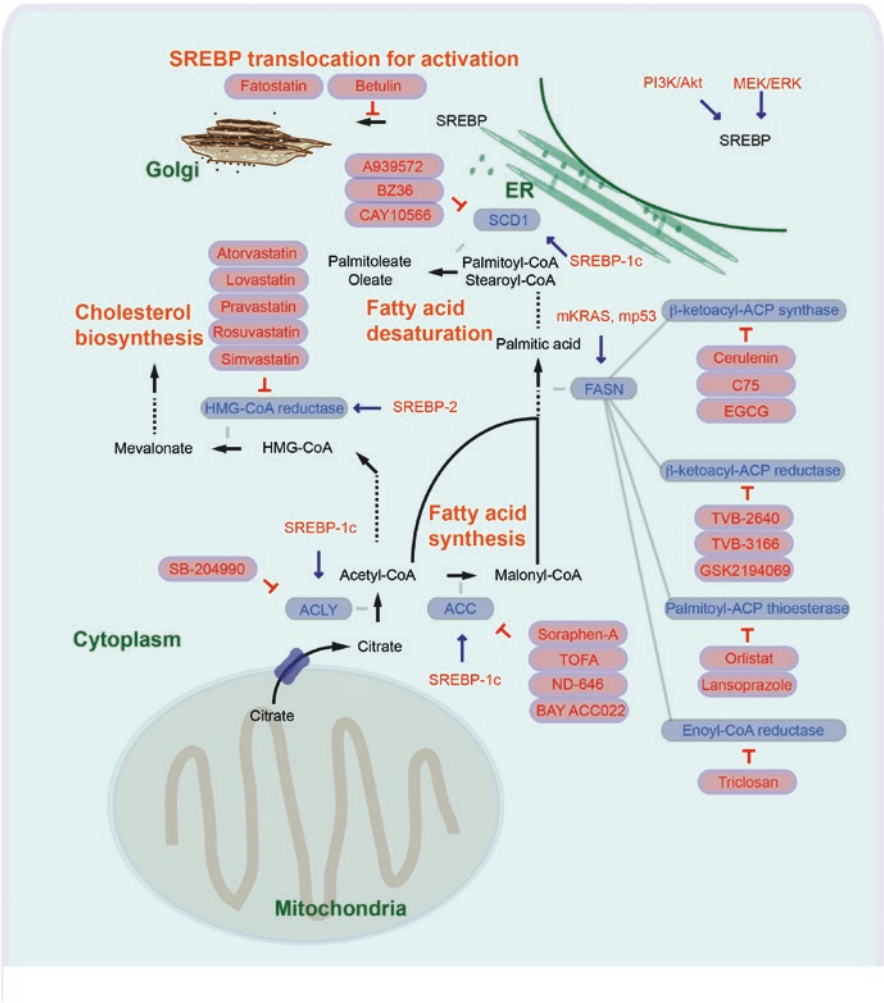

Fig. 13.2 Regulation of fatty acid synthesis, cholesterol synthesis, fatty acid desaturation, and SREBP translocation. ACC acetyl-CoA carboxylase, ACLY ATP-citrate lyase, ER endoplasmic reticulum, FASN fatty acid synthase, HMG-CoA 3-hydroxy-3-methylglutaryl-CoA or β-hydroxy-β-methylglutaryl-CoA, SCD Δ^9-stearoyl-CoA desaturase, SREBP sterol regulatory element-binding protein

NADPH-dependent manner, a basic 16-carbon saturated FA called palmitic acid is generated [45]. In cancer cells, expression of ACLY and ACC is also markedly increased [44]. Furthermore, serum FASN levels are higher in patients with PDAC, in patients with intraductal papillary mucinous neoplasm (IPMN), and in patients with chronic pancreatitis in comparison to healthy controls [46]. Pancreatic cancer patients with high FASN expression in the pancreas show a shorter overall survival than patients with low FASN expression [47]. Furthermore, FASN expression is correlated with poor response to gemcitabine therapy in pancreatic cancer cells [47, 48]. Increased *Fasn* gene expression is also observed in a pancreatic cancer mouse model with oncogenic $KRAS^{G12D}$ and p53 R172H mutation [47], suggesting that enzymes involved in fatty acid synthesis can be important targets.

Targeting Fatty Acid Synthesis in Cancer

For targeting fatty acid synthesis, several inhibitors for ACLY, ACC, and FASN blockade have been proposed. SB-204990 is an ACLY inhibitor which inhibits lipid synthesis. Intraperitoneal administration of SB-204990 leads to reduced tumor growth in mice carrying xenografts of primary mouse PDAC lines generated from oncogenic $KRAS^{G12D}$ with or without p53 R172H mutation [49]. For inhibiting ACC, soraphen A and TOFA (5-(tetradecyloxy)-2-furoic acid) have been shown to block cancer cell growth [50], and treatment with TOFA suppresses the proliferation of pancreatic cancer cells [51]. In a mouse xenograft model, it has been demonstrated that intraperitoneally administered TOFA reduces human ovarian cancer cell development [52]. Oral administration of another ACC inhibitor ND-646 suppresses FA synthesis and tumor growth in lung cancer mouse models where tumors are induced by oncogenic $KRAS^{G12D}$ with p53 deficiency or by oncogenic $KRAS^{G12D}$ with *Stk11* knockout [53]. Serine/threonine kinase 11, also known as liver kinase B1 (LKB1), activates AMPK for ACC inhibition. BAY ACC022 (another ACC inhibitor) attenuates growth of pancreatic cancer cell xenograft in mice [54]. These observations suggest that inhibiting the first step of FA synthesis is an attractive strategy for cancer therapy.

Targeting FASN can be performed by several different inhibitors, since FASN is a multienzyme protein complex with two identical polypeptides. The enzyme complex includes several catalytic domains with ACP, malonyl/acetyltransferase (MAT), β-ketoacyl-ACP synthase, β-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, enoyl-CoA reductase, and palmitoyl-ACP thioesterase. Several inhibitors block β-ketoacyl-ACP synthase of FASN, namely, cerulenin, C75 (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid, cerulenin-derived semisynthetic FASN inhibitor with improved stability), and epigallocatechin-3 gallate (EGCG) [44]. Cerulenin and C75 have been tested in several cancer xenograft models like for ovary, prostate, mesothelioma, breast, and colon cancer. Intraperitoneally administered cerulenin also suppresses liver metastasis of colon cancer cells in mice [55]. Blockage of FASN with EGCG has been considered for a broad range of cancer

types such as prostate, lung, breast, and colorectal cancer [56, 57]. EGCG inhibits pancreatic cancer cell proliferation, and antiproliferative effects are also observed with catechin gallate (CG) and epicatechin gallate (ECG) [58]. EGCG inhibits growth of pancreatic tumor cells orthotopically implanted in mice [59]. For inhibiting β-ketoacyl-ACP reductase, several compounds like TVB-2640, TVB-3166, and GSK2194069 have been proposed. TVB-2640 has entered clinical trials, e.g., for colon cancer, breast cancer, and astrocytoma. Treatment with TVB-3166 leads to inhibition of proliferation and reduction in tumor growth of multiple cancer cell lines and pancreatic cancer xenografts [57, 60]. The β-lactone orlistat blocks palmitoyl-ACP thioesterase, and enoyl-CoA reductase can be blocked by triclosan [44, 61]. Orlistat is a US food and Drug Administration (FDA)-approved anti-obesity drug, and it has been shown that orlistat reduces human pancreatic cancer cell growth [47, 62]. Inhibition of FASN with orlistat suppresses growth of EGFR tyrosine kinase inhibitor-resistant cancer cells and also tumors in EGFR mutant transgenic mice [63]. One main limitation of orlistat is its low oral bioavailability, and improved formulation of orlistat-like inhibitors may be required in the future. Alternatively, other inhibitors of palmitoyl-ACP thioesterase can be identified via in silico screening of FDA-approved drugs. Lansoprazole, rabeprazole, omeprazole, and pantoprazole are proton pump inhibitors, but also function as inhibitors of thioesterase activity, which can induce pancreatic cancer cell death [64]. In conclusion, a number of inhibitors of ACLY, ACC, and FASN have been proposed and show significant effects in cancer therapy.

Fatty Acid Desaturases: Not Just a Modifier

The main product of FA synthesis in the cytoplasm is 16-carbon saturated palmitic acid. Longer FAs are formed by reactions catalyzed by several enzymes on the cytosolic side of the endoplasmic reticulum (ER). The desaturation of fatty acids occurs also in ER membranes. These modifications support the production of a wide variety of FAs and lipids. In mammalian cells, three types of fatty acid desaturases introduce carbon double bonds at Δ^5 (Δ^5-eicosatrienoyl-CoA desaturase), Δ^6 (Δ^6-oleoyl(linolenoyl)-CoA desaturase), or Δ^9 (Δ^9-stearoyl-CoA desaturase) (SCD). SCD is the rate-limiting enzyme catalyzing the synthesis of monounsaturated 16- or 18-carbon-like palmitoleate and oleate from palmitoyl-CoA and stearoyl-CoA [65]. Enhanced FA synthesis in cancer cells also increases the requirement of enzymes for modifying FAs and lipids. SCD1 (the main isoform) has been associated with insulin resistance and diabetes. Expression of SCD1 is associated with tumor promotion, shorter survival of lung cancer patients [66], and with sorafenib resistance in liver cancer patients [67]. SCD1 expression is upregulated in human colorectal cancer tissues, and patients with high SCD1 expression levels have a shorter overall survival [68]. It has also been suggested that increased SCD1 expression is associated with shorter survival of pancreatic cancer patients [69]. SCD1 contributes to the maintenance of cancer cell stemness, and knockdown of SCD1 reduces the

expression of stemness markers like *SOX2* and *NANOG* [70]. Cancer stemness may be responsible not only for tumor initiation but also for metastasis [71]. Taken together, targeting SCD1 could be a promising option.

Targeting Fatty Acid Desaturases

However, the role of SCD1 remains controversial and requires further investigation. In a murine intestinal cancer model with a mutant allele Min (multiple intestinal neoplasia) of the Apc (adenomatous polyposis coli) locus (called $Apc^{Min/+}$ mice), conditional deletion of *Scd1* in the intestinal epithelium promotes inflammation and tumorigenesis [72]. On the other hand, the inhibitor A939572 has been applied for renal cell carcinoma treatment. Oral administration of A939572 inhibits the development of tumor xenografts in mice [73]. Intraperitoneal injection with another SCD1 inhibitor (BZ36) reduces prostate cancer xenografts in mice [74]. Furthermore, pretreatment with the SCD1 inhibitor CAY10566 suppresses ovarian tumor growth after inoculation of cancer stem cells, where inhibition of SCD1 impairs cancer cell stemness [70]. The effects these inhibitors have on pancreatic cancer cells are currently not known.

Sterol Regulatory Element-Binding Proteins: Master Regulators of Lipid Biogenesis and Cholesterol Metabolism

Expression of genes involved in FA synthesis and modification such as *ACLY*, *ACACA/B* (coding ACCs), *FASN*, and *SCD* is regulated by the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) that is itself regulated transcriptionally and/or posttranslationally by several signaling pathways and factors such as PI3K/Akt and MEK/ERK [75]. EGFR signaling is required for oncogenic $KRAS^{G12D}$-induced pancreatic tumorigenesis [76, 77], and EGFR activation also induces upregulation of FASN in pancreatic cancer cells in an ERK-dependent manner [78]. Along this line, PDAC patients with high SREBP1 expression have a shorter overall survival than patients with low SREBP1 expression, and knockdown of *SREBF1* (for SREBP1 expression) decreases pancreatic cancer cell viability and proliferation [79]. Taken together, oncogenic signaling pathways activate expression of lipogenic enzymes leading to aberrant activation of FA synthesis, which supports cancer cell development.

There are three SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2. Both SREBP-1a and SREBP-1c are derived from a single gene but through alternative transcription start sites. Whereas SREBP-1c preferentially regulates genes of FA metabolism, SREBP-1a is a potent activator of all SREBP-responsive genes, and SREBP-2 regulates cholesterol biosynthesis [80]. Cholesterol is an essential structural component of cell membranes together with various phospholipids,

sphingomyelin, and glycolipids. Cholesterol is de novo synthesized from cytoplasmic acetyl-CoA through the mevalonate pathway. The rate-limiting step of the pathway is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA, also known as β-hydroxy-β-methylglutaryl-CoA) to mevalonate by HMG-CoA reductase [81]. In addition to the mevalonate pathway, cells can increase their cholesterol contents thought receptor-mediated endocytosis of low-density lipoproteins (LDLs) [82]. The LDL receptor (LDLR) and HMG-CoA reductase are both transcriptional targets of SREBP-2 [80]. Expression of HMG-CoA reductase and LDLR is elevated in an oncogenic $KRAS^{G12D}$ pancreatic cancer mouse model [83]. It has been suggested that cholesterol intake is associated with increased risk of pancreatic cancer [84]. Increased expression of *Ldlr* has no significant effect on overall survival of pancreatic cancer patients, but high *Ldlr* expression is associated with an increased risk of tumor recurrence. Since LDLR silencing reduces ERK signaling as well as proliferation of PDAC cells, silencing also enhances response to gemcitabine chemotherapy [83].

Targeting Cholesterol Synthesis and SREBP

The development of LDLR-inactivating agents is currently an ongoing issue. Alternatively, SREBP-1c and SREBP-2 can be potential targets for cancer therapy, since these are key regulators of FASN expression and other enzymes in fatty acid synthesis like ACLY and ACC, and it also regulates expression of SCD, LDLR, and HMG-CoA reductase. SREBPs interact with the SREBP cleavage-activating protein (SCAP), and the complex stays with the ER membrane proteins INSIG1 and INSIG2. Under physiological conditions, reduction of cellular lipid levels results in conformational change of SCAP that abrogates its interaction with INSIGs. Dissociation of the SREBP/SCAP complex from INSIGs leads to transport of the complex from the ER to the Golgi where SREBP is cleaved and activated [85]. Glucose can enhance SCAP stability and reduce its association with INSIGs allowing transport of the SREBP/SCAP complex to the Golgi [86]. Betulin and fatostatin have been proposed as SREBP inhibitors through inhibition of ER-Golgi translocation. Betulin has initially been shown to improve hyperlipidemia and insulin resistance and to reduce atherosclerotic plaques [87]. Intraperitoneal injection of betulinic acid combined with mithramycin A (DNA and RNA polymerase inhibitor) blocks the development of pancreatic cancer xenografts in mice [88]. Fatostatin injection reduces expression of FASN, ACC, SCD1, ACLY, and also *Hmgcr* (HMG-CoA reductase) and *Ldlr* transcription to a lesser extent in obese mice [85]. The inhibitor has been tested in glioblastoma and prostate cancer cell xenografts. There, intraperitoneal treatment with fatostatin reduced xenograft growth in mice [89, 90]. Inhibiting de novo cholesterol synthesis by blockage of the rate-limiting enzyme HMG-CoA reductase has also been considered for cancer therapy. Several statin derivatives such as atorvastatin, lovastatin, pravastatin, rosuvastatin, and simvastatin have entered clinical trials. Among the derivatives, atorvastatin and simvastatin

have been considered for pancreatic cancer treatment [57]. Taken together, there are several therapeutic options targeting SREBP and the mevalonate pathway, and a number of cancer studies are currently ongoing.

Glutamine and Acetate Metabolism in Pancreatic Cancer

Glutamine Metabolism: It Works Also Without Mitochondria

By modulating the activity of several metabolic pathways including glutamine metabolism, cancer cells aim for continuous generation of FAs necessary for cell growth. Glutamine is the most abundant and nonessential amino acid that can be synthesized from glucose. In the canonical route of mitochondrial glutamine catabolism (glutaminolysis), glutaminase (GLS) catalyzes glutamine to glutamate (Fig. 13.3). Glutamate is further converted by glutamate dehydrogenase (GLUD1)

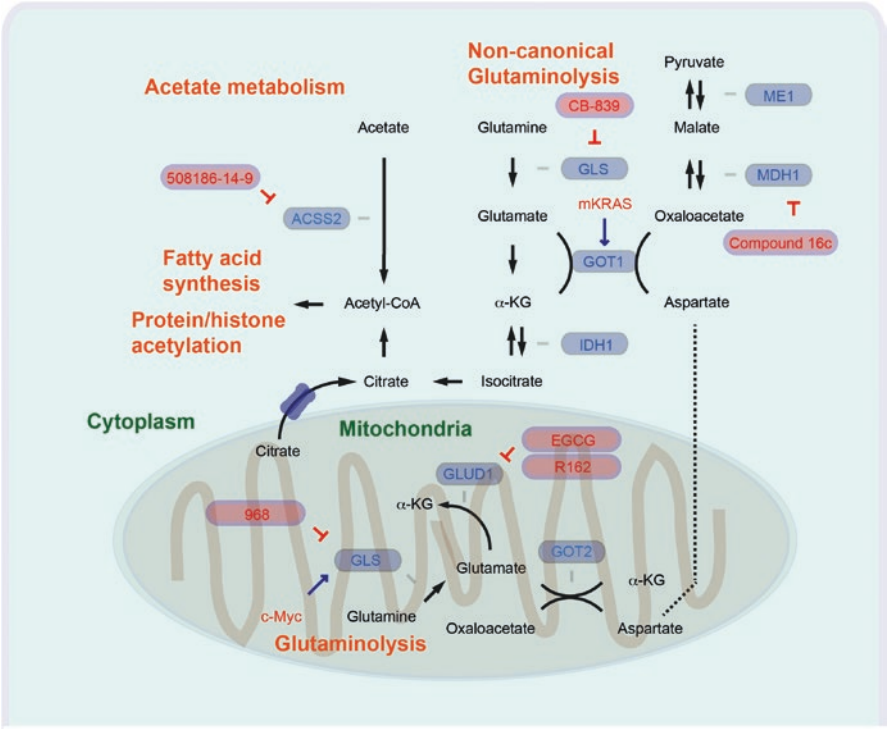

Fig. 13.3 Regulation of glutaminolysis and acetate metabolism. ACSS short-chain acyl-CoA synthetase, GLS glutaminase, GLUD glutamate dehydrogenase, GOT aspartate transaminase, IDH isocitrate dehydrogenase, MDH malate dehydrogenase, ME malate enzyme

to α-ketoglutarate (α-KG), and α-KG can then be integrated into the tricarboxylic acid cycle (TCA cycle). Glutamine is an essential nutrient for the proliferation of human cancer cells [91], and several oncogenes which activate glutaminolysis have been identified. Oncogenic c-Myc enhances expression of mitochondrial GLS supporting canonical glutaminolysis [92]. Pancreatic cancer cells rely on a cytoplasmic noncanonical glutaminolysis pathway producing pyruvate via aspartate transaminase (GOT1, catalyzes aspartate/oxaloacetate), malate dehydrogenase (MDH1, catalyzes malate/oxaloacetate), and malate enzyme (ME1, catalyzes malate/pyruvate). Oncogenic KRAS induces a shift from canonical to noncanonical glutaminolysis by inhibiting mitochondrial GLUD1 and activating cytoplasmic GOT1 [93]. By reprogramming of glutamine metabolism from the mitochondrial to the cytoplasmic system, pancreatic cancer can keep synthesis of FAs intact, because cytoplasmic isocitrate dehydrogenase (IDH1) can catalyze α-KG/isocitrate under hypoxic conditions or even with defective mitochondria [94–96].

Targeting Glutamine Metabolism

Several drugs such as 968, BPTES, and CB-839 have been developed to inhibit GLS glutamate synthesis. Treatment with 968 or with BPTES reduces pancreatic cancer cell viability [93]. Intravenous injection of BPTES nanoparticles reduces pancreatic cancer xenograft growth in mice, and combination with intraperitoneal injection of metformin enhances therapeutic effects [97]. CB-839 has already been tested in several clinical studies including a broad range of cancer types, such as clear cell renal carcinoma, breast cancer, and colorectal cancer. However, oral gavage of CB-839 has no antitumor activity in mice with oncogenic $KRAS^{G12D}$ combined with Trp53 deficiency. In addition, mice treated with CB-839 show marginally shorter survival than the group without CB-839 treatment [98]. Further investigations are therefore required to judge whether GLS inhibition is a potential therapeutic option for pancreatic cancer patients. EGCG and R162 have been considered to inhibit GLUD1 [99]. EGCG has been described as a FASN β-ketoacyl-ACP synthase inhibitor and shown to inhibit pancreatic cancer cell proliferation (see Targeting Fatty Acid Synthesis in Cancer), and it is also recognized as a GLUD1 inhibitor. Treatment with R162 inhibits proliferation of several cancer cells including primary leukemia cells. Furthermore, intraperitoneal injection of R162 inhibits the development of lung cancer xenografts in mice [100]. Oncogenic $KRAS^{G12D}$ has been suggested to inhibit GLUD1 and preferentially activate the noncanonical glutaminolysis pathway (see Glutamine Metabolism: It Works Also Without Mitochondria); thus, GLUD1 inhibition might be ineffective in pancreatic cancer. Methyl 3-(3-(4-(2,4,4-trimethylpentan-2-yl)phenoxy)-propanamido)benzoate (named compound 16c) has been synthesized to inhibit the noncanonical glutaminolysis pathway as a MDH inhibitor. This inhibitor blocks both cytoplasmic MDH1 and mitochondrial MDH2 enzymes. It has been shown that intraperitoneal administration of this inhibitor attenuates the development of colon cancer xenografts [101]. Since inhibition of

MDH1 activity leads to suppression of glutamine metabolism and reduction of pancreatic cancer cell growth [102], inhibitors for the noncanonical glutaminolysis pathway could be potential candidates for pancreatic cancer therapy.

Acetate Metabolism: Cancer Cells Are Experts in Bridging the Gap

Acetyl-CoA represents a central metabolite not only for lipid synthesis but also for regulating gene expression as a key determinant of protein/histone acetylation [103, 104]. Cancer cells preferentially convert pyruvate into lactate rather than to transport it into the mitochondria for PDH reaction and the TCA cycle. Although the IDH1-mediated non-canonical glutaminolysis pathway (see Glutamine Metabolism: It Works Also Without Mitochondria) may compensate to provide acetyl-CoA in the cytoplasm, alternative sources of acetyl-CoA could still be necessary for sufficient supporting lipid synthesis and cancer cell growth. Cells with ACLY deficiency remain viable and proliferate, where acetate supports acetyl-CoA generation and de novo lipid synthesis is supported by the enzyme called ACSS2 [105]. There have been 26 acyl-CoA synthetases (ACS) identified in the human genome. Among those, three enzymes, the short-chain ACS (ACSS) family (acetyl-CoA synthetase), are capable of catalyzing synthesis of acetyl-CoA from acetate in an ATP-dependent manner [106]. ACSS1 and ACSS3 are mitochondrial enzymes, and ACSS2 localizes to both the cytoplasmic and nuclear compartments. Silencing of ACSS2 in cancer cells reduces incorporation of acetyl units from acetate into either lipids or histones. ACSS2 is highly expressed in several human tumors, and loss of ACSS2 suppresses tumor development in certian mouse liver cancer models including c-Myc combined with PTEN knockout [107]. Under metabolic stress such as hypoxia and/or low-nutrition conditions, expression of ACSS2 is elevated, and it promotes acetate uptake for lipid synthesis and membrane phospholipids in several cancers including pancreatic cancer cells [108, 109].

Inhibitors specifically targeting ACSS2 remain largely unexplored. So far a compound 1-(2,3-di(thiophen-2-yl)quinoxalin-6-yl)-3-(2-methoxyethyl)urea (PubChem CID: 2300455; here referred to as 508186-14-9) has been proposed as a ACSS2-specific inhibitor [107]. The inhibitor has been tested and showed decreased lipid contents in bladder cancer cells, but not in non-cancer cells [110]. Targeting ACSS2 and acetate metabolism would be a highly interesting concept for treating pancreatic cancer.

Conclusion

Extensive research on cancer metabolism has revealed that a number of enzymes and metabolites are involved in reprogramming strategies of many cancer types including pancreatic cancer. Furthermore, it is evident that overexpression of

specific enzymes is not only related with metabolic reprogramming but also with cellular stemness. Several studies with inhibitors targeting specific catalyzing steps in selected metabolic pathways have shown convincing effects in inhibiting cancer development and progression. Cancers may however still find other ways to generate necessary metabolic intermediates and cellular components. Therefore, it is important to further understand not only the cross talk between oncogenic signaling pathways and metabolism but also between metabolic pathways for offering stratified and more effective therapies in the future.

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Chapter 14
Targeting the Immune System in Pancreatic Cancer

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The Immune Response in Pancreatic Cancer and Its Major Players

The immune system can be both harmful and beneficial during carcinogenesis and progression of pancreatic cancer (PC). The ability of both innate and adaptive immune cells to exert either tumor-suppressive or tumor-promoting properties yields a mosaic pattern of immune cell composition in the tumor microenvironment (TME). Therefore, an understanding of the individual components of this mosaic is required to develop efficient therapeutics.

Chronic inflammation is an important characteristic of PC, which is maintained by a complex interplay of immune cells in the TME [1, 2]. The myeloid compartment has many components, undoubtedly the most important one of them being tumor-associated macrophages (TAM). TAMs are found as M1 or M2 macrophages, which are classified according to the cytokine profile and surface markers they express [3]. Both M1 and M2 macrophages derive from monocytes. M1 macrophages, as "good cops," produce pro-inflammatory cytokines like TNF, IL12, IL-1β, and IFN-γ and show tumoricidal activity and induce an antitumor Th1 immune response. On the other hand, M2 macrophages, as the "bad cops," produce anti-inflammatory tumor-promoting cytokines like TGFβ and IL-10 and stimulate a Th2 immune response [3]. Next to TAMs, myeloid-derived suppressor cells (MDSC) are produced from immature myeloid cells and are known to suppress

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adaptive immunity with the recruitment of regulatory T cells (T_{reg}) to the TME and by reducing antitumor T cell activation [4, 5]. In line with this, the presence of immunosuppressive cells like M2 macrophages, MDSCs, and T_{reg} cells in the pancreatic TME has been shown to negatively correlate with overall survival [6–11]. Although both pro- and antitumorigenic abilities of neutrophils are reported, the inhibition of neutrophil recruitment to the TME remains a promising option in preclinical studies [12–14].

In the adaptive immune system, antigen-presenting cells (APC) such as dendritic cells (DC) can prime naïve T cells broadly into functional $CD4^+$ helper T cells (Th) or $CD8^+$ cytotoxic T cells (CTL) [15]. Th cells are further mainly characterized as Th_1, Th_2, and T_{reg}, and their coordination is highly deterministic for the type of tumor immune response [15]. Th_1 cells as conductors of an antitumorigenic response promote antigen presentation on APCs and cytolytic activity of $CD8^+$ T cells and boost M1 macrophages [16, 17]. However, Th_2 and T_{reg} cells are pro-tumorigenic since they can oppose the Th_1 immune response and escalate T cell exhaustion. Their presence is correlated with reduced survival in PC patients [18–23]. $CD8^+$ CTLs are the "best cops" in tumors, since they can directly recognize tumor cell-specific antigens and induce cancer cell death [15, 24].

Immunotherapy for PC: Obstacles and Potential Solutions

Boosting the adaptive immune response is one of the most attractive goals in cancer therapeutics: Other than generating a repertoire of T cells recognizing tumor-specific antigens, the ability of the adaptive immune system to form an immunological memory holds promise for long-term disease control [25]. Immunotherapeutic approaches, currently being established as a fourth pillar of cancer therapeutics (next to chemo-/targeted therapy, radiotherapy, and surgery), augment the antitumor adaptive immune response [26]. Immune checkpoint inhibitors are the best studied candidates in immunotherapeutic options so far. While checkpoint inhibitors like anti-CTLA-4 and anti-PD-1 antibodies showed very promising results in clinical studies for many solid tumors and hematologic malignancies, as single agents or in combination, they appear to be ineffective in PC [27–36]. Therefore, precise understanding of the immune cell network in PC is essential to explore ways to exploit immunotherapeutic approaches for treatment of patients with PC.

Immune Checkpoint Inhibition

CTLA-4 and PD-1 were the first immune checkpoint targets discovered and evaluated for cancer immunotherapeutics [37–39]. During APC:MHC molecule engagement with T cell receptor (TCR) on T cells, axes of co-stimulatory and co-inhibitory signals in T cells mediate T cell activity. These co-signaling pathways are essential

for physiological homeostasis since an imbalance can cause either autoimmunity or disability to fight invaders. Tumors may evolve the ability to skew this balance by reducing co-stimulation and inducing co-inhibition to impair antitumor T cell activity [40]. CTLA-4 and PD-1 are such co-inhibitory molecules leading to T cell anergy and exhaustion [41–44]. Antibodies targeting CTLA-4 and PD-1 can impair such signaling pathways in T cells and boost an antitumor cytotoxic immune response in tumors.

The question is though, why checkpoint inhibitors are not effective in PC as opposed to other solid tumor entities. PC owes this to its extreme immune-privileged nature [45]. Immune privilege is the ability to retain the production of antigens, without creating an anti-tumor immune response [46]. Normally, during carcinogenesis, tumor cells produce unique antigens (de novo mutations, re-expression of embryonic stage proteins), which may be recognized by the immune system, potentially leading to tumor cell elimination. During the immunosurveillance process (a hypothesis developed by Paul Ehrlich), the immune system continuously inspects the body for any malignant transformation [47–49]. However, some transformed cells have the ability to escape detection in a process called immunoediting. Immunoediting proposed by Schreiber and colleagues comprises three phases (triple E): elimination, equilibrium, and escape [50]. During the elimination phase, most of the transformed somatic cells die due to immunosurveillance, while the remaining survivors in the equilibrium step no more respond to immune reaction. Through a Darwinian-like selection, these clones proliferate and expand within the escape phase. While many tumors undergo the triple E of immunoediting process, PC holds a unique state [51, 52].

PC carcinogenesis is different in terms of the immunoediting process compared to many other solid tumors. With the use of genetically engineered mouse models (GEMMs), PC was shown to have an immunosuppressive microenvironment and a scarcity of antitumor T cells already during the carcinogenesis process [45]. Due to immunosuppression, the adaptive immune system is not educated toward recognition of any tumor-specific antigens, bypassing the elimination phase of triple E. With this rather immune quiescence-like phenotype, PC limits the entry of antitumor immune cells into the microenvironment maintaining its immune privileged status [51].

Overall, an approach to augment T cell entry and activity in the PC microenvironment may have the ability to render PC cells responsive toward immune checkpoint inhibitors. The factors which will determine such responsiveness are (1st) antigenicity of cancer cells and (2nd) immunogenicity of the tumor in general [53].

Antigenicity is the degree to which tumor cells produce and present neoantigens to generate an antitumor adaptive immune response [53]. These antigens can be divided into tumor-specific antigens (TSA) and tumor-associated antigens (TAA). TSAs are produced upon tumor-specific mutations of genes or reactivation of genes for embryonic development, which are not occurring in healthy somatic cells, while TAAs are wild-type proteins but expressed higher in tumor cells compared to somatic ones [54]. Production and MHC-mediated presentation of such antigens determine the level of antigenicity of tumors [53, 54].

Tumors carrying a high mutational burden generally respond better to checkpoint inhibition since they have a diverse tumor-antigen responsive T cell repertoire [55–57]. PC on the other hand doesn't carry such mutational load, compared to other entities [58, 59]. However, a subgroup of PC patients, representing around 1% of a patient cohort, carry mutations leading to mismatch repair (MMR) deficiency and microsatellite instability (MSI) and may profit from checkpoint inhibitors [60, 61]. As a result, anti PD-1 immunotherapy is approved by FDA for solid tumors including PC with MMR deficiency and MSI [62]. Moreover, one study identified long-term survivors in a PC patient cohort based on their ability to express good quality neoantigens, but not quantity [63]. Most importantly, a decrease in neoantigen quality of metastatic tumors compared to their respective primaries implied the importance of immunosurveillance in cancer metastasis and its implication in therapeutics [63]. Other than antigen production, presentation of these antigens via MHC molecules has been shown to be reduced in PC through the activation of oncogenic drivers like RAS [64–66]. Also, reduced MHC expression in disseminated PC cells appears to be an important driver of metastasis [67]. Since a correlation between antigenic load and immune checkpoint inhibition efficacy is absent in PC, as opposed by other solid tumor entities, in addition, factors determining immunogenicity of PC require exploitation.

Tumors with better ability to induce an adaptive immune response are considered immunogenic. This ability can be modulated both at the tumor cell level and at the level of cross talk of tumor cells with cells of the TME [53]. Transcriptomic analyses revealed an immunogenic subtype of PC, showing higher cytolytic T cell activity, antigen presentation, and CTLA-4 and PD-1 signatures [68]. Signatures as those may help to predetermine the prognostic value of checkpoint inhibitor therapy in the context of "personalized medicine" [69].

Tumor cell-specific immunogenicity can be decreased upon co-inhibitory checkpoint ligand expression in tumor cells, such as PD-L1. In various solid tumors, PD-L1 expression by tumor cells is increased due to oncogenic signaling pathways like PI3K, Hippo, Myc, and JAK-STAT [70–74]. In PC, the myeloid compartment was shown to induce EGFR-dependent MAPK signaling, leading to an increase of PD-L1 production in tumor cells [75]. An imbalance of autophagic modulation in mitochondrial iron homeostasis also may induce PD-L1 expression by pancreatic cancer cells [76].

Reprogramming the Tumor Microenvironment

Even if specific cancer cells are sufficiently antigenic and immunogenic, they may still not respond well to checkpoint inhibition due to an overall impaired immunogenicity mediated by the corresponding tumor tissue. The immunosuppressive TME is the main player in this context. An understanding of the responsible TME compartments, and of their cross talk with antitumor adaptive immune cells, is essential to reveal options for boosting immune checkpoint inhibitor response (Fig. 14.1).

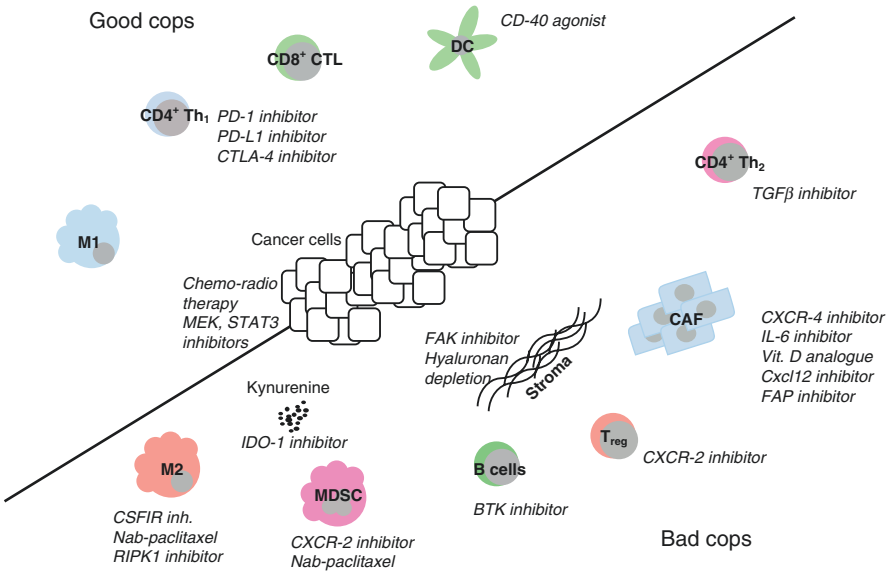

Fig. 14.1 The good and the bad cops of the tumor microenvironment and how to target them to boost a favorable immune response in PC. M1: M1 macrophages, M2: M2 macrophages, MDSC: myeloid-derived suppressor cells, CTL: cytotoxic T lymphocytes, DC: dendritic cells, T_{reg}: regulatory T cells, CAF: cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are the leading actors regarding the characteristic desmoplastic stroma formation in PC. Various studies revealed a binary action of stromal cells in the immunogenicity of PC. One study revealed a positive correlation between type-I collagen production and CTL infiltration in tumor specimens of PC patients, whereas another showed the inhibition of CTL activity by αSMA$^+$ CAFs [77]. Other studies demonstrated an inhibitory action of CAFs toward CD8$^+$ T cell infiltration [78]. While most of the research so far implies the prognostic value of "stromal remodeling" in PC, an understanding of CAF action heterogeneity in the TME may provide options to improve the efficacy of immune checkpoint inhibitors. For example, with the use of preclinical mouse models, impairment of CXCR4 or IL-6 signaling in CAFs was shown to be synergistic with anti PD-L1 therapy [79, 80]. Stromal remodeling with FAK inhibitors reduced the immunosuppressive milieu in the TME, increasing chemotherapy-checkpoint inhibitor combination therapy efficacy [81]. Previous studies showed the benefit of hyaluronan depletion and vitamin D receptor activation in stromal remodeling [82–85]. Here, a combination therapy with immune checkpoint inhibitors may have therapeutic impact.

The myeloid compartment is a double-edged sword as also mentioned earlier. Years of research dissected the complex roles of individual components in PC. Studies focusing on CD40 agonist treatment of PC actually revealed the quite unique properties of PC. Treatment of preclinical mouse models with a CD40 agonist (acting on APCs increasing their capability to prime CTL) in combination with

gemcitabine created an only mild response by remodeling the stroma and reprogramming immunosuppressive myeloid cells inside the TME [86]. However, this regimen was not enough to create an adaptive immune response in tumors. The subsequent studies identified a subtype of immunosuppressive macrophages ($Ly6C^{low}$ $F4/80^{+}$), accumulating in the tumor periphery. These macrophages were shown to prevent CTL migration into the TME [87]. Finally, a combination therapy of nab-paclitaxel with gemcitabine and CD40 agonist revealed a synergism allowing penetration of active CTLs [88].

Re-education of neutrophils, MDSCs, and TAMs can also be achieved via various inhibitors targeting CSF1R, CXCR2, or RIPK1, which demonstrated synergism with immune checkpoint inhibitors in preclinical studies [14, 89, 90]. Other than directly targeting the myeloid compartment, inhibition of B cell-specific Bruton's tyrosine kinase (BTK) reprogrammed tumor resident macrophages indirectly, increasing the antitumor immunity [91].

Immunosuppressive immune cells impair immunosurveillance not only via cytokine-chemokine release but also through generation of a metabolite-restricted TME. Arginine depletion via arginase-1 produced by TAMs and MDSCs limits T cell activity [92, 93]. Further, the immunosuppressive metabolite kynurenine is produced from tryptophan as a by-product of indoleamine 2,3-dioxygenase (IDO-1) enzymatic activity. IDO-1 expression from cancer cells, TAMs, and MDSCs not only limits tryptophan availability for antitumor T cells but also increases inhibition of T cell activity by kynurenine [94]. Adenosine production by T_{reg} cells and prostaglandin E2 production from TAMs and MDSCs are also responsible for antitumor T cell activity impairment [95, 96].

Immunotherapeutic Properties of "Classical" Treatment Approaches

Other than targeted inhibitors, chemotherapeutic agents and radiotherapy also have the ability to convert nonresponsive, "immunologically cold," tumors to responsive, "immunologically hot," tumors. Chemo- and radiotherapy can boost both, antigenic properties of cancer cells due to their mutagenic effect and also immunogenicity of the tumor due to the induction of immunogenic cell death and subsequently enhanced inflammation [97, 98]. Next to their direct effect on cancer cells, such treatments may also alter the composition of immunosuppressive immune cells in the TME [88, 99]. Strikingly, immune checkpoint inhibition in cancer may not only enhance the response to radiation therapy in primary tumors but also has the potential for an abscopal response in metastatic sites [100, 101]. In conclusion, while chemotherapy and radiotherapy still are the gold standard therapies for cancer treatment, their combination with checkpoint inhibitors may be the next step to both increase the treatment response and T cell memory for long-term disease control, even for PC. Essentially, analysis of respective clinical trials may inform about dosing, sequence of treatment, and specific subgroups profiting most from the expected synergism.

Other Strategies for Boosting the Antitumor Immune Response

Immunotherapeutic approaches are not only limited to immune checkpoint inhibitors.

Oncolytic viruses (OV) can be designed to only target tumor cells, but not healthy somatic ones. This specificity can be achieved at multiple levels [102]. At the physiological level, OVs are not equipped to win a combat against healthy cells. Tumor cells, however, already may have imbalanced interferon signaling and increased cellular metabolism coupled with proliferation making them vulnerable towards viral infection. OVs can also be designed to take advantage of tumor-specific expression of cell entry receptors or transcription factors, limiting their action on healthy cells.

Cancer vaccines aim to boost adaptive immune response in the host against tumors. They can be produced as either whole cell (e.g. GVAX) or antigen-specific vaccines. GVAX is composed of pancreatic cancer cells genetically engineered to secrete GM-CSF with the aim to convert "cold" tumors to "hot" ones, and these cells are irradiated to prevent further proliferation [103]. *Listeria* vaccine is an engineered bacterial strain to secrete TAAs such as human mesothelin, boosting antitumor CTL activity. An approach with total cell followed by antigen-specific vaccine may recapitulate a "prime and boost" scenario [104].

Chimeric antigen receptor T cells (CAR-T) are genetically designed to express a receptor construct comprising an antibody-like ectodomain targeting TSAs and a TCR-like endodomain, bypassing the need for MHC engagement [105]. Upon antigen recognition they exert their cytotoxic properties. CAR-T cell therapy requires adoptive T cell transfer (ATC), in which patient's T cells have to be isolated, expanded, and genetically engineered. Without a genetic manipulation, in vitro induction and expansion of TILs (TIL-ATC) is also a valuable approach to exploit tumor targeting not only by a single antigen but a pool of them [106, 107].

Currently Ongoing Clinical Trials for Immunotherapy of Patients with PC

An overview of clinical trials based on abovementioned preclinical studies is given in Table 14.1. Overall, these studies reveal that PC is actually antigenic enough to create an antitumor adaptive immune response. However, the main barrier to be exceeded is the immunosuppressive microenvironment, which blocks the antitumor T cell priming and infiltration. One important factor is that many of these studies for PC are still in their early stages. Thorough analysis of each of these trials will pave the way to dissect individual rationales for combination therapies.

Table 14.1 Selected clinical trials aiming to induce an antitumor immune response in pancreatic cancer

Combination-arm 1	Combination-arm 2	Status	Patient eligibility criteria	Trial ID
Ipilimumab (αCTLA-4), gemcitabine	–	Phase 1	Stage III–IV or recurrent pancreatic cancer, uneligible to surgery	NCT01473940
Nab-paclitaxel, gemcitabine, nivolumab (αPD-1)	Nab-paclitaxel and nivolumab	Completed/ phase 1	Multiple solid tumors including pancreatic cancer	NCT01473941
Cyclophosphamide, GVAX, pembrolizumab (αPD-1), radiation (SBRT-6.6 Gy)	–	Recruiting/ phase 2	Locally advanced pancreatic ductal adenocarcinoma upon standard chemotherapy	NCT02648282
Durvalumab (αPD-L1), radiation (SBRT-6.6 Gy)	–	Recruiting/ phase 1–2	Borderline resectable and locally advanced pancreatic adenocarcinoma, treated with standard of care (SOC)	NCT03245541
Cyclophosphamide, GVAX, nivolumab (αPD-1), radiation (SBRT-6.6 Gy)	–	Recruiting/ phase 2	Borderline resectable pancreatic cancer	NCT03161379
Durvalumab (αPD-L1), radiation (SBRT-6.6 Gy)	–	Recruiting/ phase 1–2	SOC treated, borderline resectable, and locally advanced pancreatic adenocarcinoma	NCT03245541
Durvalumab (αPD-L1), tremelimumab (αCTLA4), radiation (SBRT-6.6 Gy)	Radiation (SBRT-6.6 Gy) with either durvalumab or tremelimumab	Recruiting/ phase 1	Uunresectable, nonmetastatic, locally advanced adenocarcinoma of pancreas	NCT02868632
Avelumab (αPD-L1), binimetinib (MEK inhibitor), talazoparib (PARP inhibitor)	Avelumab, binimetinib	Recruiting/ phase 2	Locally advanced or metastatic Ras-mutant solid tumors, including pancreatic cancer	NCT03637491
Durvalumab (αPD-L1), AZD9150 (STAT3 antisense)	–	Recruiting/ phase 2	Advanced pancreatic cancer	NCT02983578
Pembrolizumab (αPD-1), paricalcitol (vit D analogue)	Pembrolizumab, placebo	Recruiting/ early phase 2	Stage IV pancreatic cancer	NCT03331562

Table 14.1 (continued)

Combination-arm 1	Combination-arm 2	Status	Patient eligibility criteria	Trial ID
PEGPH20 (hyaluronidase), pembrolizumab (αPD-1)	–	Phase 2	Hyaluronan high (HA-high) metastatic pancreatic ductal adenocarcinoma	NCT03634332
PEGPH20 (hyaluronidase), avelumab (αPD-L1)	–	Recruiting/ early phase 1	Chemotherapy-resistant advanced or locally advanced pancreatic ductal adenocarcinoma	NCT03481920
Galunisertib (TGFβ inhibitor), durvalumab (αPD-L1)	–	Phase 1	Metastatic pancreatic cancer	NCT02734160
Spartalizumab (αPD-1), NIS793 (TGFβ inhibitor)	NIS793 (TGFβ inhibitor)	Recruiting/ phase 1	Advanced malignancies including pancreatic cancer	NCT02947165
Pembrolizumab (αPD-1), defactinib (FAK inhibitor)	–	Recruiting/ phase 1–2	Advanced solid malignancies including pancreatic neoplasms	NCT02758587
Pembrolizumab (αPD-1), defactinib (FAK inhibitor), gemcitabine	–	Recruiting/ phase 1	Advanced solid malignancies including pancreatic cancer	NCT02546531
Pembrolizumab (αPD-1), defactinib (FAK inhibitor)	Pembrolizumab (αPD-1)	Recruiting/ phase 2	SOC treated, neoadjuvant, and adjuvant treatment for resectable pancreatic ductal adenocarcinoma	NCT03727880
Cyclophosphamide, GVAX, pembrolizumab (αPD-1), IMC-CS4 (CSF1R inhibitor)	–	Recruiting/ early phase 1	Borderline resectable pancreatic ductal adenocarcinoma	NCT03153410
Durvalumab (αPD-L1), pexidartinib (CSF1R, FLT3, and KIT inhibitor)	–	Recruiting/ phase 1	Metastatic/ advanced pancreatic or colorectal cancers	NCT02777710
Nivolumab (αPD-1), cabiralizumab (αCSF1R)	Cabiralizumab	Phase 1	Advanced solid tumors including pancreatic cancer	NCT02526017

(continued)

Table 14.1 (continued)

Combination-arm 1	Combination-arm 2	Status	Patient eligibility criteria	Trial ID
Pembrolizumab (αPD-1), AMG820 (CSF1R inhibitor)	–	Phase 1–2	Advanced solid tumors including pancreatic cancer	NCT02713529
Pembrolizumab (αPD-1), BL-8040 (CXCR4 inhibitor)	BL-8040	Phase 2	Metastatic pancreatic adenocarcinoma	NCT02826486
Olaptesed pegol (CXCL12 inhibitor) + Pembrolizumab	Olaptesed pegol	Phase 1–2	Metastatic colorectal and pancreatic cancer	NCT03168139
APX005M (CD40 agonist), gemcitabine, nab-paclitaxel, nivolumab (αPD-1)	APX005M, gemcitabine, nab-paclitaxel	Recruiting/ phase 1–2	Previously untreated metastatic pancreatic adenocarcinoma	NCT03214250
CDX-1140 (CD40 agonist), CDX-301 (CD135 agonist)	CDX-1140	Recruiting/ phase 1	Advanced malignancies including pancreatic adenocarcinoma	NCT03329950
Pembrolizumab (αPD-1), acalabrutinib (BTK inhibitor)	Acalabrutinib	Phase 2	Metastatic pancreatic cancer	NCT02362048
Durvalumab (αPD-L1), ibritunib (BTK inhibitor)	–	Completed/ phase 1–2	Relapsed or refractory solid tumors including pancreatic cancer	NCT02403271
Epacadostat (IDO-1 inhibitor), pembrolizumab (αPD-1)	–	Phase 2/ withdrawn	Advanced pancreatic cancer with chromosomal instability/ homologous recombination repair deficiency (HRRD)	NCT03432676

Table 14.1 (continued)

Combination-arm 1	Combination-arm 2	Status	Patient eligibility criteria	Trial ID
Atezolizumab (αPD-L1), chemotherapy, selicrelumab (CD40 agonist)	Nab-paclitaxel, gemcitabine (chemotherapy)	Recruiting/ phase 1–2	Cohort 1 treatment to be performed on patients with no prior systemic therapy for metastatic pancreatic ductal adenocarcinoma	NCT03193190
Atezolizumab (αPD-L1), chemotherapy, selicrelumab (CD40 agonist), bevacizumab (αVEGF)				
Atezolizumab (αPD-L1) + chemotherapy + bevacizumab (αVEGF)				
Atezolizumab (αPD-L1) + chemotherapy + emactuzumab (αCSF1R)				
Atezolizumab (αPD-L1) + cobimetinib (MEK inhibitor)	Nab-paclitaxel and gemcitabine or mFOLFOX6 (chemotherapy)		Cohort 2 treatment to be performed on patients with disease progression upon control chemotherapy of cohort 1	
Atezolizumab (αPD-L1) + PEGPH20 (hyaluronidase)				
Atezolizumab + BL-8040 (CXCR4 inhibitor)				
Atezolizumab (αPD-L1) + RO6874281 (FAP-IL2 fusion protein)				
Atezolizumab (αPD-L1) + emactuzumab (αCSF1R)				

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Chapter 15
Phase I Trials in Pancreatic Cancer

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For a long time pancreatic ductal adenocarcinoma (PDAC) has been a largely neglected entity in clinical research. Only recently there has been a substantial increase in the number, but also the spectrum of clinical trials for the treatment of pancreatic cancer in different clinical settings. This is partly due to a better understanding of the molecular setup of pancreatic cancer and consequently the definition of subgroups that allow a more specific targeting.

In this chapter we will highlight recent trends in the very early phase of clinical trials in pancreatic cancer. There are substantial activities in targeting specific signaling pathways overexpressed or active in PDAC, the cell cycle, and DNA damage repair, but also the tumor microenvironment including the stromal compartment and the immune system. Given the still poor prognosis of pancreatic cancer, even rather novel approaches such as CarT cells are tried in pancreatic cancer.

Interfering with Signaling Pathways

Targeting Receptor Tyrosine Kinases

"Classical" approaches targeting receptor tyrosine kinases are still examined in phase I trials for PDAC. The PDGFRa inhibitor olaratumab is examined in combination with gemcitabine plus nab-paclitaxel in metastatic PDAC (mPDAC) (NCT03086369).

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HER2 3+ overexpression and/or gene amplification is observed in about 10% of patients with pancreatic cancer [1]. A previous study using trastuzumab in patients with pancreatic cancer exhibiting overexpression of the human epidermal growth factor receptor 2 (HER2) did not demonstrate a benefit for adding trastuzumab to chemotherapy. A phase I/II trial examines a novel approach. A166 is an antibody drug conjugate composed of a monoclonal antibody (mAb) targeting HER2 and conjugated to a cytotoxic agent that has not been disclosed so far. A166 is examined in locally advanced/metastatic solid tumors including PDAC with HER2 expression or amplification (NCT03602079).

The HGF/c-MET signaling module plays a major role in the interaction between pancreatic stellate cells and the tumor cells in pancreatic cancer. Ficlatuzumab, a mAb that binds soluble HGF and thereby interrupts this interaction [2], is currently examined in combination with nab-paclitaxel and gemcitabine in patients with previously untreated PDAC (NCT03316599).

Overexpression of B-type ephrins correlates with progression of PDAC and is involved in angiogenesis and tumor growth. Currently, a recombinant EphB4-HSA fusion protein (sEphB4-HSA) is under investigation in a multi-arm study in combination with various chemotherapy regimens in patients with advanced solid tumors including PDAC. sEphB4-HAS is a recombinant fusion protein composed of the extracellular domain of human receptor tyrosine kinase ephrin type-B receptor 4 (sEphB4) and fused to full-length human serum albumin (HSA) [3]. sEphB4-HSA acts a decoy receptor for the membrane-bound ligand ephrin-B2 (Efnb2) and interferes with the binding of Efnb2 to its native receptors, including EphB4 and EphA3 (NCT02495896).

The TGF-beta/TGF-beta receptor type 1 (TGFBR1) signaling module is highly expressed in many PDACs and plays a major role in tumor formation and metastases. A phase Ib trial evaluates safety, tolerability, and exploratory efficacy of vactosertib (TEW-7197) in combination with FOLFOX in the second-line treatment of patients with mPDAC (NCT03666832). Vactosertib is an orally bioavailable inhibitor of TGFBR1 (also known as activin receptor-like kinase 5 (ALK5)) serine threonine kinase activity. Since activation of the TGFBR1 can also suppress the response of the host immune system to tumor cells, another trial examines safety and tolerability of the anti-TGF-beta antibody NIS793 either alone or in combination with an immune checkpoint inhibitor, PDR001 (NCT02947165).

The receptor tyrosine kinase Axl also plays a role in invasion and metastasis of PDAC. Bemcentinib is a small molecule tyrosine kinase inhibitor that inhibits Axl by binding to its intracellular catalytic kinase domain [4]. It is examined in combination with nab-paclitaxel/gemcitabine/cisplatin in patients with mPDAC. A goal of this study is to determine the complete response rate of bemcentinib plus chemotherapy in these patients (NCT03649321).

Another drug that targets the Axl pathway is BA3011. BA3011 is a conditionally active biologic AXL-targeted antibody drug conjugate (CAB-AXL-ADC) that has been designed to reversibly bind to recombinant AXL and AXL-expressing cells under conditions that are only present in the tumor microenvironment, but not in normal tissues. The study evaluates safety, tolerability, pharmacokinetics (PK),

immunogenicity, and antitumor activity of BA3011 in patients with advanced solid tumors including PDAC (NCT03425279).

Fusions involving one of the three tropomyosin receptor kinases (TRK) rarely occur in pancreatic cancer but make the respective tumors highly susceptible to TRK inhibitors [5]. However, during treatment with a TRK inhibitor, acquired resistance can occur, particularly due to kinase domain mutations. LOXO-195 can overcome resistance in TRK fusion-positive cancers with an acquired kinase domain mutation [6]. A current phase 1/2, multicenter, open-label study evaluates safety and efficacy of LOXO-195 in patients with NTRK fusion cancers including PDAC treated with a prior TRK inhibitor (NCT03215511).

The glucocorticoid receptor is frequently overexpressed in PDAC [7]. Furthermore, dexamethasone that is regularly used as supportive treatment during chemotherapy has been implicated in tumor proliferation, chemotherapy resistance, and metastasis [8]. CORT125134 is a glucocorticoid receptor (GR) antagonist that is examined in combination with nab-paclitaxel in patients with solid tumors including PDAC to determine safety and efficacy of this combination (NCT02762981).

Apart from receptor tyrosine kinases, there are multiple intracellular pathways that contribute to tumor progression, invasion, metastasis, and the communication between PDAC cells and their microenvironment.

Protein Kinase Inhibitors

Focal adhesion kinase (FAK) is overexpressed and active in pancreatic cancer [9]. Recently, it could be demonstrated that FAK plays a key role in the regulation of the fibrotic and immunosuppressive microenvironment [10]. Inhibition of FAK is hypothesized to make tumors responsive to checkpoint inhibitors and could delay tumor progression in combination with chemotherapy and checkpoint inhibitors. A current phase I trial examines the combination of the FAK inhibitor defactinib in combination with the checkpoint inhibitor pembrolizumab and gemcitabine in patients with advanced solid tumors including pancreatic cancer (NCT02546531).

The Ras-MEK-ERK cascade is active in PDAC, partly due to the constitutively active $KRAS^{G12D}$, partly due to overexpression of receptor tyrosine kinases and their respective ligands and other factors, respectively. Therefore, interfering with this signaling cascade may have antiproliferative effects. Several phase I trials examine novel ERK inhibitors such as BVD-523 in combination with nab-paclitaxel and gemcitabine in patients with mPDAC (NCT02608229) or the ERK inhibitor ASN007 in patients with advanced solid tumors including PDAC (NCT03415126).

GSK-3β is a potentially important therapeutic target in human malignancies. The kinase is involved in energy metabolism, neuronal cell development, and body pattern formation [11]. Aberrantly active GSK3b can mediate tumor invasion and treatment resistance [12]. This has led to the design of GSK3b inhibitors for clinical use. A phase I/II study evaluates the safety and efficacy of 9-ING-41, a potent GSK-3β

inhibitor, as a single agent and in combination with cytotoxic agents in patients with refractory cancers including PDAC (NCT03678883).

ABTL0812 is a small molecule that activates the nuclear receptors PPARα/γ and thereby induces the pseudokinase TRIB3 which in turn leads to inhibition of the Akt/mTORC1 axis and induces autophagy-mediated cancer cell death [13]. A current trial examines the efficacy and safety of ABTL0812 in combination with gemcitabine and nab-paclitaxel in patients with mPDAC (NCT03417921).

The mTOR pathway is also target of another phase I study that evaluates MLN0128 or sapanisertib, an experimental small molecule inhibitor of mTOR, in combination with ziv-aflibercept (NSC# 724770) in patients with advanced cancers including PDAC (NCT02159989).

Phosphoinositide-3 kinases (PI3Ks), upstream regulators of AKT and mTOR, play a key role in tumor-associated immune responses, tumor cell growth, survival, proliferation, angiogenesis, and dissemination as well as tumor-stroma cross talk [14]. INCB050465 inhibits the delta isoform of PI3K and is currently examined alone and in combination with the checkpoint inhibitor pembrolizumab in advanced solid tumors including pancreatic cancer (NCT02646748).

Wnt signaling also plays a role in certain pancreatic cancers [15]. A phase I trial investigates LGK974, a potent and specific inhibitor of porcupine, a central component of the Wnt pathway, in patients with various malignancies dependent on Wnt ligands (NCT01351103).

Inhibiting Mutated Kras

The small GTP binding protein KRAS is frequently mutated in pancreatic cancer but as yet regarded as not druggable. Recently, a novel approach has been described using modified small extracellular vesicles, so-called exosomes, that are produced by mesenchymal stromal cells and have been engineered to contain shRNA against KRAS with a G12D mutation (iExosomes). These exosomes have been shown to be delivered to the tumor and block growth of $Kras^{G12D}$-mutated PDACs [16]. A current phase I trial examines side effects and the best dose of iExosomes in treating patients with metastatic $Kras^{G12D}$-mutated PDAC (NCT03608631).

Inhibition of Mutated KRAS Signaling by Protein Phosphatase Inhibitors

Apart from protein kinases, their counterparts, protein phosphatases, emerge as interesting targets in PDAC. Recent data show that mutant KRAS-driven cancers depend on PTPN11/SHP2 phosphatase [17]. A current phase I study examines oral RMC-4630, a protein tyrosine phosphatase non-receptor type 11/SHP2 antagonist [18], as monotherapy in patients with advanced relapsed or refractory solid tumors harboring mutations/rearrangements that result in hyperactivation of the RAS-MAPK pathway (NCT03634982).

Targeting Heat Shock Proteins (HSPS)

Heat shock proteins act as molecular chaperones responsible for proper folding and activation of their substrate proteins. They are ubiquitously expressed and have been implicated in tumor cell proliferation, invasion, metastasis, and cell death [19]. Therefore, HSPs constitute promising targets.

Minnelide is a prodrug of triptolide and has been derived from the thunder God vine (*Tripterygium wilfordii*) [20]. One of its mechanisms of action is inhibition of HSP70. A phase I trial evaluates dose, safety, pharmacokinetics, and pharmacodynamics (PD) of this compound in patients with advanced solid tumors including PDAC (NCT03129139). A further phase I trial examines an HSP90 inhibitor, XL888, when given together with the checkpoint inhibitor pembrolizumab in treating patients with advanced metastatic gastrointestinal cancers including PDAC (NCT03095781).

Cell Cycle Inhibitors

The cell cycle is an attractive target in cancer. Various clinical trials investigate the use of cell cycle inhibitors in order to improve the treatment of patients with PDAC. A phase I/II clinical trial assesses the maximum tolerated dose, safety, and efficacy of BEY1107, an inhibitor of the CDK1 protein kinase, as monotherapy and in combination with gemcitabine in patients with locally advanced or metastatic PDAC (NCT03579836).

LY3143921 hydrate inhibits the serine/threonine kinase CDC7 that regulates chromosomal DNA replication. CDC7 is overexpressed in pancreatic cancer and inhibition of CDC7 results in apoptosis of pancreatic cancer cells [21]. The compound is examined in patients with advanced solid tumors including PDAC (NCT03096054).

Another phase I study evaluates safety, tolerability, and pharmacokinetics of SBP-101 in combination with nab-paclitaxel and gemcitabine in patients with mPDAC (NCT03412799). SBP-101 is a polyamine (PA) analogue that displaces endogenous PAs from PA-binding sites on the cell surface and thereby prevents internalization of PA which in turn blocks cell cycle progression. This may be even a tumor-specific mechanism of action since PA uptake is upregulated in various tumor types and increased levels of PA result in enhanced tumor cell growth.

Furthermore, combinations of selective inhibitors targeting different signaling pathways are evaluated. A current phase I study assesses safety and MTD of the ERK inhibitor ulixertinib (BVD-523) combined with the CDK4/6 inhibitor palbociclib (NCT03454035). Palbociclib is also combined with the PI3K/mTOR inhibitor gedatolisib (PF-05212384) for patients with advanced solid tumors including PDAC (NCT03065062).

DNA Damage Repair as Target

Targeting DNA damage repair has become an interesting approach in cancers with particular vulnerabilities such as mutations in the BRCA1 and BRCA2 genes. These tumors respond well to platinum-based chemotherapies and to poly(ADP-ribose) polymerase (PARP) inhibitors. PARP1 thereby is highly relevant in repairing DNA single-strand breaks. BRCA1 and BRCA2 mutations are comparatively rare in PDAC being detectable only in 1–4% of PDACs in a general population.

BTP-114 is a cisplatin prodrug with a maleimide moiety that strongly and selectively binds human serum albumin in the bloodstream prolonging the half-life and improving the biodistribution of the drug [22]. A phase I trial evaluates BTP-114 in patients with advanced solid tumors and BRCA or other DNA repair mutation (NCT02950064).

A single-arm phase I/II study examines the clinical activity of a novel PARP inhibitor, ABT-888, in combination with modified FOLFOX-6 (5-fluorouracil plus oxaliplatin) in patients with metastatic PDAC (NCT01489865). Another trial investigates the effectiveness, safety, and antitumor activity of the PARP inhibitor niraparib with either ipilimumab, a mAb against CTLA-4, or the PD-1 mAb nivolumab in patients with PDAC whose disease has not progressed on a platinum-based therapy (NCT03404960).

Also combinations of PARP inhibitors with chemotherapy are evaluated. A randomized phase II study assesses the combination of gemcitabine, cisplatin +/−, the PARP inhibitor veliparib in patients with PDAC, and a known BRCA/PALB2 mutation. In a second part of this trial, veliparib is examined as monotherapy (NCT01585805).

Histone Deacetylase (HDAC) Inhibitors

Targeting epigenetic regulation in solid tumors is an upcoming strategy that is studied in various tumor entities including PDAC. The majority of trials examines class I HDAC inhibitors either alone or in combination with chemotherapy or immunotherapy, respectively: The HDAC inhibitor CG200745 PPA is evaluated in combination with gemcitabine and erlotinib (NCT02737228), the HDAC1 and HDAC3 inhibitor entinostat in combination with FOLFOX (NCT03760614).

Super-Enhancers (SEs) as Targets

SEs are unique areas of the genome that are densely bound by numerous transcription factors. SEs often drive high-level transcription. Many genes that play an important role in cancer biology are likely to be SE-driven oncogenes [23, 24]. A phase I trial investigates the SE inhibitor GZ17-6.02 in patients with advanced solid tumors including PDAC (NCT03775525). GZ17-6.02 is a synthetic formulation of *Arum palaestinum* extracts that has shown antitumor activity against PDAC [24].

Targeting Tumor Metabolism

Due to the high genomic heterogeneity of PDAC, approaches have been sought in order to allow efficient treatment of these heterogeneous tumors. One of these approaches is addressing key metabolic pathways in PDAC.

RGX-202-01 is a small molecule inhibitor of the creatine transporter solute carrier family 6, member 8 (SLC6a8). RGX-202-01 reduces the intracellular levels of phosphocreatine available for ATP synthesis in tumor cells, thereby limiting tumor cell growth and metastasis [25]. The compound is examined with or without FOLFIRI (NCT03597581).

Tumor cell pyruvate dehydrogenase and alpha-ketoglutarate of the TCA cycle are inhibited using a lipoate analog, CPI-613, in a clinical trial. The compound mimics lipoate, a catalytic cofactor for both enzymes, and thereby inactivates the two enzymes. Tumor specificity is thought to result from the fact that many tumor cells overexpress a distinct set of lipoate-sensitive regulators. CPI-613 is examined in combination with gemcitabine and nab-paclitaxel in PDAC (NCT03435289).

The enzyme NAD(P)H dehydrogenase [quinone] 1 is encoded by the *NQO1* gene that encodes the enzyme 2-electron reductase. The NQO1 inhibitor ARQ 761, an intravenously administered analogue of naturally occurring β-lapachone, is examined in a phase I/Ib trial in combination with gemcitabine plus nab-paclitaxel in metastatic and locally advanced PDAC (NCT02514031).

Induction of Apoptosis

RX-3117 is an oral, small molecule nucleoside prodrug that is activated/phosphorylated by uridine-cytidine kinase 2 (UCK2). UCK2 is predominantly expressed in cancer cells. Once activated, it is incorporated into the DNA or RNA of cancer cells and induces apoptotic cell death. Because UCK2 is overexpressed in multiple human tumors, RX-3117 may be a comparatively selective nucleoside analogue. In a phase I trial, RX-3117 is examined in combination with gemcitabine plus nab-paclitaxel (NCT03189914).

A phase Ib/II trial studies the side effects and best dose of the Bcl-2 inhibitor navitoclax in combination with the MEK1/MEK2 inhibitor trametinib in patients with metastatic solid tumors including PDAC (NCT02079740).

GEN1029 (HexaBody®-DR5/DR5) is an agonistic hexamer formation-enhanced mixture of two antibodies (HexaBody) that target two separate epitopes on death receptor type 5 (DR5; TNFRSF10B; tumor necrosis factor-related apoptosis-inducing ligand receptor 2; TRAILR2) and has potential antineoplastic activity [26]. Upon administration, DR5 HexaBody agonist GEN1029 specifically binds to and activates DR5. A current first in human phase I trial examines this compound also in patients with advanced PDAC (NCT03576131).

Targeting the Cytoskeleton

Fascin is an actin filament bundling protein that is also a biomarker of invasive and advanced PDAC and regulates PDA cell migration and invasion in vitro. NP-G2-044 is a fascin inhibitor that is examined in a first-in-human phase I study to determine its safety when given orally (NCT03199586) [27].

Anetumab ravtansine or BAY 94-9343 is an antibody-drug conjugate consisting of a human anti-mesothelin antibody conjugated to the maytansinoid tubulin inhibitor DM4. The antibody binds selectively to mesothelin on cancer cells, and upon internalization the DM4 moiety disrupts microtubule assembly/disassembly dynamics, thereby inhibiting cell division. A phase Ib study examines this compound in patients with mesothelin expressing advanced or recurrent malignancies including PDAC (NCT03102320).

Targeting the Microenvironment

Stroma

The human cytokine leukemia inhibitory factor (LIF) is overexpressed in PDAC and drives PDAC-associated neural remodeling. In addition, LIF has immunosuppressive properties in cancer. MSC-1 is a first-in-class, humanized monoclonal antibody (IgG1) that binds LIF [28]. A current trial evaluates the safety and antitumor activity of MSC-1 in patients with solid tumors including PDAC (NCT03490669).

Hyaluronic acid is a major component of the tumor stroma in PDAC. Pegylated hyaluronidase (PEGP20) can improve permeability of the tumor stroma as well as tumor vascularization in PDAC, thereby improving the penetration of chemotherapeutic agents [29]. A current trial examines pharmacodynamics, safety, and efficacy of PEGPH20 in combination with the anti-PD-L1 mAb avelumab in adult patients with chemotherapy-resistant, advanced PDAC (NCT03481920).

The vitamin A derivative all-trans retinoic acid (ATRA) may also have the ability to break down stroma allowing chemotherapy to reach the cancer. A study examines the combination of ATRA, gemcitabine, and nab-paclitaxel in patients with locally advanced or metastatic PDAC (NCT03307148).

Immunotherapeutic Approaches

Chimeric Antigen Receptor (CAR) T Cells

Chimeric antigen receptor T cell (CAR-T) therapy is beginning to be explored in solid tumors. Numerous trials examine various antigens as a CAR-T cell approach in PDAC including CEA-targeted CAR-T cells (NCT02349724); anti-HER2

CAR-modified T cells (NCT02713984); EpCAM-specific CAR-T cells for EpCAM-positive cancers (NCT03013712); CAR-T cells that target mesothelin, given as single agent or in combination with a lymphocyte depleting dose of cyclophosphamide (NCT03323944); or anti-KRAS G12V mTCR cells (NCT03190941). There are also trials testing various antigens such as mesothelin, PSCA, CEA, HER2, MUC1, and EGFRvIII for CAR-T cell immunotherapy for PDAC (NCT03267173).

Further Immunotherapies

Oleclumab (MEDI9447) is a human mAb that binds to CD73/5′-nucleotidase and inhibits the production of adenosine and its immunosuppressive properties [30]. A phase I trial evaluates safety, antitumor activity, and immunogenicity of oleclumab with or without the checkpoint inhibitor durvalumab in combination with chemotherapy (gemcitabine plus nab-paclitaxel or mFOLFOX) in patients with mPDAC in the first and second line setting (NCT03611556).

CPI-006 is a type 2 humanized IgG1 antibody that inhibits the enzymatic activity of CD73 and adenosine production. A trial investigates safety, tolerability, and antitumor activity of CPI-006 as a single agent, in combination with CPI-444, a small molecule targeting the adenosine-A2A receptor on immune cells, and in combination with pembrolizumab, an anti-PD1 antibody against various solid tumors (NCT03454451).

Receptor-interacting serine/threonine-protein kinase 1, or RIPK1, regulates macrophages. Inhibition of RIPK1 results in a doubling of killer T cell activation and a fivefold decrease in the macrophage-influenced T cell type that suppresses the immune system [31]. A phase I/II study examines safety, clinical activity, pharmacokinetics, and pharmacodynamics of the RIPK1 inhibitor GSK3145095 alone and in combination with pembrolizumab in advanced solid tumors including PDAC (NCT03681951).

ADCT-301 or camidanlumab tesirine combines HuMax®-TAC™, a monoclonal antibody targeting CD25 (the alpha chain of the IL-2 receptor) with a highly potent pyrrolobenzodiazepine (PBD)-based warhead. In preclinical in vivo models, ADCT-301 exhibits strong dose-dependent antitumor activity against CD25-positive cell lines including cancer cells at low single doses [32]. A phase Ib trial evaluates safety, tolerability, pharmacokinetics, and antitumor activity of ADCT-301 in patients with advanced solid tumors including pancreatic cancer (NCT03621982).

FATE-NK100 is a first-in-class natural killer (NK) cell cancer immunotherapy comprised of adaptive memory NK cells, a highly specialized and functionally distinct subset of natural killer cells. A phase I study examines FATE-NK100 as monotherapy in patients with advanced solid tumors and also in combination with trastuzumab in case of HER2+ or in combination with cetuximab in patients with EGFR1+ advanced solid tumors (NCT03319459).

Immune checkpoint inhibitors, especially PD1/PD-L1 inhibitors, have only very limited efficacy as single agents in PDAC unless the tumors exhibit high microsatellite instability. Nevertheless, novel anti-programmed cell death ligand 1 (PD-L1)

checkpoint antibodies such as LY3300054 are examined in patients with advanced refractory solid tumors including PDAC in phase I trials (NCT02791334).

Alternatively activated (M2-type) macrophages may protect tumor cells from cytotoxic T cells and thereby confer resistance to PD1/PD-L1 targeted agents in PDAC. Blocking CSF1R to deplete the tumor microenvironment of M2 macrophages may enable a more robust cytotoxic antitumor T cell response following PD-L1 blockade and sensitize PDAC to this approach. A phase I trial examines the combination of an anti-CSF1R (pexidartinib) with an anti-PD-L1 mAb (durvalumab) in patients with advanced/metastatic PDAC (NCT02777710).

Another sensitizing approach is the combination of immune checkpoint inhibitors with co-stimulatory molecules. T cell activation induces co-stimulatory molecules, including the ICos (inducible co-stimulator). ICos belongs to the CD28 family and is only expressed at low levels on naive T cells. ICos-mediated signals contribute mainly to the regulation of activated T cells and to effector T cell functions [33]. A phase I trial examines XmAb23104, a bispecific anti-PD1 and anti-ICOS antibody in subjects with selected advanced solid tumors including PDAC (NCT03752398).

It has been demonstrated that CD40 agonists can alter the stroma and inhibit growth of PDAC. RO7009789 is a novel CD40 agonist antibody with potential antineoplastic and immunostimulatory properties [34]. A clinical trial examines neoadjuvant RO7009789 alone or in combination with nab-paclitaxel and gemcitabine followed by adjuvant RO7009789 plus nab-paclitaxel and gemcitabine for patients with newly diagnosed, resectable PDAC (NCT02588443).

Another approach to reverse immunosuppression in pancreatic cancer is targeting macrophage infiltration mediated by the CCL2/CCR2 axis. The CCR5/CCL5 chemokine axis also promotes migratory and invasive properties of PDAC [35]. A phase I trial evaluates safety, PD, and preliminary efficacy of the CCR2/CCR5 antagonist BMS-813160 alone or in combination with chemotherapy or nivolumab in patients with metastatic colorectal and pancreatic cancers (NCT03184870).

Therapeutic Viruses

A phase I study examines intravenous administration of the VCN-01 oncolytic adenovirus with or without gemcitabine and nab-paclitaxel in patients with advanced solid tumors including PDAC. VCN-01 is a replication-competent adenovirus that expresses PH20 hyaluronidase that targets hyaluronic acid, a major component of the PDAC stroma (NCT02045602) [36].

Another phase I trial examines of the tolerability and safety of a replication-competent adenovirus-mediated double suicide gene therapy (Ad5-yCD/mutTKSR39rep-ADP) in combination with chemotherapy for locally advanced PDAC (LAPC) (NCT02894944).

TBI-1401(HF10) is a replication-competent HSV-1 oncolytic virus that is studied in combination with chemotherapy (gemcitabine + nab-paclitaxel or TS-1) in Japanese patients with stage III or IV unresectable PDAC (NCT03252808).

A phase I/IIa trial evaluates intratumoral injection of LOAd703, an armed oncolytic adenovirus, in combination with gemcitabine and nab-paclitaxel in patients with PDAC (NCT02705196).

Targeting Specific Antigens and Vaccination Strategies

TAK-164 is an antibody-drug conjugate comprising a full-length, fully human IgG1 monoclonal antibody (mAb) directed toward the extracellular domain of guanylyl cyclase C (GCC) [37]. TAK-164 binds to antigen-expressing cells resulting in a GCC-dependent uptake and cytotoxicity. A phase I study examines TAK-164 in patients with advanced GI malignancies including PDAC (NCT03449030).

NEO-201 is a humanized IgG1 mAb derived from an immunogenic preparation of tumor-associated antigens from pooled allogeneic colon tumor tissue extracts. It reacts against a wide variety of human tumor tissues, but is largely nonreactive against normal tissues [38]. NEO-201 binds to members of the CEACAM family and can activate innate immune mechanisms such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. A first-in-human phase I trial determines safety and DLT of NEO-201 in patients with advanced solid tumors including PDAC (NCT03476681).

A phase Ib/II trial examines the NANT vaccine as treatment for patients with advanced PDAC. A combination of agents will be administered to subjects in this study: aldoxorubicin HCl, ALT-803, ETBX-011 (CEA), ETBX-021 (HER2), ETBX-051 (brachyury), ETBX-061 (MUC1), GI-4000, GI-6207, GI-6301, haNK for infusion, avelumab, bevacizumab, capecitabine, cyclophosphamide, fluorouracil, leucovorin, nab-paclitaxel, oxaliplatin, and stereotactic body radiation therapy (SBRT) (NCT03586869).

CV301 is a targeted MUC1 and CEA vaccination strategy that is examined in a phase I/II study in combination with durvalumab and maintenance chemotherapy in patients with metastatic PDAC whose disease is stable on or responding to first-line therapy for metastatic disease (NCT03376659).

The combination of cyclophosphamide, pembrolizumab, GVAX, and IMC-CS4, a CSF1R blocking antibody, is examined in patients with borderline resectable pancreatic cancer (NCT03153410).

MVT-5873 is a fully human IgG1 monoclonal antibody (mAb) that targets sialyl Lewis A (sLe^a), an epitope on CA19-9 which is expressed in PDAC and other GI cancers, plays a role in tumor adhesion and metastasis, and is a marker of an aggressive tumor phenotype [39]. A phase I trial evaluates MVT-5873 as monotherapy and in combination with a standard of care chemotherapy in patients with PDAC (NCT02672917).

A carboanhydrase IX (CAIX) inhibitor, SLC-0111, is examined in combination with gemcitabine in CAIX-positive mPDACs (NCT03450018).

Mesothelin is targeted in PDAC by various approaches. A phase Ib/II study examines the mesothelin-targeted immunotoxin LMB-100 alone or in combination

with nab-paclitaxel in patients with advanced PDAC and mesothelin-expressing solid tumors (NCT02810418).

APN401 is an autologous cellular therapy consisting of ex vivo cbl-b-silenced PBMCs using siRNA. Silencing of the cbl-b ubiquitin ligase in PBMCs enhances T cell and NK cell antitumor activity in mouse tumor models and in vitro in human immune cells [40]. APN401 is evaluated in patients with solid tumors including PDAC (NCT03087591).

The data presented above demonstrate that all state-of-the-art concepts currently available for cancer treatment are nowadays examined in PDAC. Of course, the phase I/Ib design is merely focused on safety, PK, and PD, and therefore many trials do not focus in this setting on biomarker or particular PDAC subgroups. However, there is an increasing number of trials that already take specific properties of PDAC such as immunosuppression into account and try to target it, focus on specific antigens, or examine a concept only in a particular subgroup (e.g., BRCA mutated tumors). This shows that even at the level of phase I/Ib studies data from basic science and translational research are much more taken into account, and we are moving into "targeted strategies" examining safety, PD, and PK of compounds or their respective combinations in specific PDAC subgroups that have a higher chance of responding to a particular treatment. This will speed up drug research and safe costs and most importantly bring true innovations faster to our patients.

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Chapter 16
Translational Approaches in Surgical Treatment

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Pancreatic ductal adenocarcinoma (PDAC) carries one of the poorest overall prognosis of all human malignancies. The 5-year survival in patients with PDAC, for all stages, remains as low as 6–7%. The low survival rate is attributed to several factors, of which the two most important are aggressive tumor biology and late stage at which most patients are diagnosed. Only 10–20% of patients are eligible for resection at presentation, 30–40% are unresectable/locally advanced, and 50–60% are metastatic [1].

Pancreatic cancer without distant metastasis can be divided into three categories: resectable, borderline resectable, and locally advanced. In absence of metastatic disease, the most important factor for improving survival and possibly offer cure is to achieve a margin-negative resection. Even after potential curative resection, most patients develop recurrences eventually, and 5-year survival of completely resected patients is only up to 25% [1]. The aggressive tumor biology and its inherent resistance to chemotherapy and radiotherapy contributes to early recurrence and metastasis.

Surgical Advances/Techniques

Pancreatic cancer surgery has evolved over the past few decades and remains the cornerstone of treatment of resectable and borderline resectable tumors. Advances in modern imaging give precise information on disease extension and vascular involvement that aids in surgical planning in order to achieve a margin-negative resection.

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Surgical techniques for pancreatic cancer include pancreaticoduodenectomy, distal pancreatectomy with splenectomy, and total pancreatectomy. Standard lymphadenectomy for pancreatoduodenectomy should include removal of lymph node stations 5, 6, 8a, 12b1, 12b2, 12c, 13a, 13b, 14a, 14b, 17a, and 17b.

Involvement of superior mesenteric vein (SMV)/portal vein(PV) was previously considered as a contraindication for resection. However, curative resection along with SMV/PV with vascular reconstruction has now become a standard practice in specialized high-volume centers. To improve margin-negative resections, specially in borderline resectable tumors with proximity to vascular structures, SMA first approach (six different approaches) was proposed as a new modification of standard pancreaticoduodenectomy [2]. In a systematic review, SMA first approach was shown to be associated with better perioperative outcomes, such as blood loss, transfusion requirements, pancreatic fistula, delayed gastric emptying, and reduced local and metastatic recurrence rates [3, 4].

In case of arterial involvement, there is no good evidence at present to justify arterial resections for right-sided pancreatic tumors [5]. However, the modified Appleby procedure, which includes en bloc removal of celiac axis with or without arterial reconstruction, when used in appropriately selected patients, offers margin-negative resection with survival benefit for locally advanced pancreatic body and tail tumors and should be performed in high-volume centers [6].

Most evidence does not support advantage of more extended resections such as removal of the para-aortic lymph nodes and nerve plexus and multivisceral resections routinely [7–9]. Such extended resections are associated with compromised quality of life because of associated higher perioperative morbidity and intractable diarrhea. However, in highly selected patients, with preserved performance status and stable or nonprogressive disease on neoadjuvant treatment, such extended resections can provide survival advantage over palliative treatments [10]. Radical surgery in the presence of oligometastatic disease has also been reported to prolong survival in highly selected patients [11].

Translational Approaches in Surgery

Currently, the AJCC (American Joint Committee on Cancer) TNM staging is the only prognostic factor used in clinical practice to assess the survival of a resected PDAC and guide treatment decisions. However, this clinicopathological staging fails to consistently predict the outcomes after pancreatic resection. Due to the large genomic heterogeneity within PDAC tumors, prognostic gene expression signatures may be useful to predict outcome.

Earlier studies had shown that the most frequently altered genes in PDACs are KRAS, SMAD4, TP53, and CDKN2A/B (one oncogene and three tumor suppressor genes) [12–14]. Many genes were later found altered by using comprehensive genomic approaches including array-comparative genomic hybridization [15, 16].

Molecular Classification of PDAC

More recently, molecular classification according to gene expression and genomic alterations has been proposed [17–19]. The first such profiling of PDAC was published in 2011 based on microdissection performed on surgically resected specimens [17]. According to the results, PDAC was classified into three different subtypes (Collison's subtypes: "classical," "quasi-mesenchymal," and "exocrine-like"). These subtypes had different clinical outcomes and therapeutic responses and were also validated externally. The classical tumor subtype had a better survival, whereas the quasi-mesenchymal subtype had worst survival. Subtype classification was the only independent prognostic factor for overall survival (OS) in multivariate analysis and the chemosensitivity also varied among the subtypes. In another study, Moffitt et al. [18] separated the stromal component from the malignant epithelial component and identified different subtypes, based on the observation that PDAC is comprised of a dense peritumoral stroma. Two specific stromal subtypes, "normal" and "activated" stroma, were identified, with the latter showing the worst prognosis (median survival of 15 months vs. 24 months). The malignant component was further classified as "classical" and "basal-like" tumor-specific subtypes. Classical tumor and normal stroma subtypes correlated with best prognosis, and prognosis was worst with basal-like tumor and activated stroma subtypes. More recent transcriptional classification for PDAC by Bailey et al. [19] distinguished four tumor subtypes associated with different molecular pathways as "squamous," "pancreatic progenitor," "immunogenic," and "aberrantly differentiated endocrine exocrine (ADEX)." This classification is based on the differential expression of transcription factors and downstream targets important for lineage specification and differentiation during pancreas development and regeneration. Correlating with outcomes, the squamous subtype was an independent poor-prognostic factor.

Indeed, identifying such genetic signatures and their expression profiling is presently the most promising approach for identifying new prognostic tools and tailoring individualized treatment in PDAC, possibly independent of the AJCC staging.

Early Detection

Late stage at diagnosis is one of the most important factors for overall dismal outcomes in PDAC. Early detection at stage I or II can provide a window of opportunity when the disease can be eradicated by high-quality surgery and together with adjuvant chemotherapy and can result in cure [20]. Development of promising molecular biomarkers for early detection of PDAC is hence the need of the hour. For this purpose, blood-based molecular biomarkers, which include proteins, nucleic acids, autoantibodies, aberrantly glycosylated antigens, exosomes, circulating tumor cells, and metabolites, have been studied. The ideal, noninvasive biomarkers should be universally present in precancerous lesions (PanIN, pancreatic

intraepithelial neoplasia; IPMN, intraductal papillary mucinous neoplasm with dysplasia; carcinoma in situ) and should have a high sensitivity and specificity which is inexpensive, rapid, and practical to perform. Current clinical practice uses CA19-9, which is a carbohydrate antigen found on multiple carrier proteins [21]. However, it is not detectable in 5–10% of patients and lacks specificity as it is often elevated in biliary obstruction with or without malignancy. Hence, it is useful for monitoring response to therapy, but it is not a useful tool as an early detection biomarker. With molecular profiling of PDAC, a number of novel biomarkers have been discovered and are under evaluation. Also, with development of organoids recapitulating PDAC, new biomarker discovery is enhanced [22].

Circulating tumor cells (CTCs) could represent another source of blood-based molecular profiles. CTCs are tumor cells that are shed off from a primary tumor into the circulation and can be detected in the blood samples (liquid biopsy) [23]. Recently, CTCs have been studied as a potential biomarker for PDAC [24]. In this study, the authors evaluated CTC subtypes (triploid, tetraploid, or multiploid cells) and their total number and found that both were upregulated in the peripheral blood of PDAC patients when compared with healthy controls, serving thus as a diagnostic tool for the disease.

Although at present these biomarkers have not been able to make a great clinical impact, the progress made to date in finding biomarkers for early detection specially in high-risk individuals (e.g., family history of PDAC, recent-onset diabetes, chronic pancreatitis, etc.) provides optimism to the field.

Chronic Pancreatitis

Chronic pancreatitis (CP) represents a risk factor for pancreatic cancer and is a frequent differential diagnosis as well [25]. CP can involve the whole pancreatic gland or can result in development of an inflammatory head mass, which can become a considerable source of diagnostic confusion, as even high-quality CT/MRI scans fail to conclusively differentiate between the two. A positive endoscopic ultrasound (EUS) or image-guided biopsy confirms presence of a cancer; however, a negative report does not conclusively rule out malignancy. In order to enhance the diagnostic accuracy of PDAC in the background of CP, molecular markers on EUS-FNA samples have been evaluated in recent years. Utilities of DNA mutations such as kras [26], p53 [27], telomerase activity with a ribonucleoprotein enzyme [28], and a broad panel of microsatellite allele loss markers [29] have been shown to improve diagnostic accuracy in such situations.

Recently metabolic biomarkers have also been studied and introduced in this field. One such study evaluated nine metabolites [proline, sphingomyelin (d18:2,C17:0), phosphatidylcholine, isocitrate, sphinganine-1-phosphate, histidine, pyruvate, ceramide, sphingomyelin (d17:1,C18:0)] along with CA 19.9 in patients with CP having high risk for PDAC and were found to have a sensitivity of 89.9% and a specificity of 91.3% for detection of maliganacy [30].

Utilization of these molecular and metabolic biomarkers may reduce the diagnostic delay and early diagnosis of PDAC in CP and can result in early initiation of treatment and surgery in resectable patients leading to improved overall outcomes.

Summary

Given the potential clinical correlation of PDAC molecular subtyping and long-term survival, the emphasis now should be on defining a universally accepted PDAC molecular subtyping which can guide personalized therapy including surgery, irrespective the AJCC stage of the disease. Also, the focus should be on formulating an ideal biomarker for early detection of PDAC, at least in high-risk population and those with chronic pancreatitis, in order to offer early curative treatment resulting in overall improved outcomes.

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